Social experience and pheromone receptor activity reprogram behavioral switch and neuromodulatory gene expression in sensory neurons

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Abstract

Social experience and pheromone signaling in olfactory neurons affect neuronal responses and male courtship behaviors in *Drosophila*. We previously showed that social experience and pheromone signaling modulate chromatin around behavioral switch gene *fruitless*, which encodes a transcription factor necessary and sufficient for male behaviors. Fruitless drives social experience dependent modulation of courtship behaviors and pheromone responses in sensory neurons, however, the molecular mechanisms underlying this neuromodulation remain less clear. To identify the molecular mechanisms driving social experience-dependent neuromodulation, we performed RNA-seq from antennal samples of mutants in pheromone receptors and *fruitless,* as well as grouped or isolated wild-type males. We found that loss of pheromone detection differentially alters the levels of *fruitless* exons suggesting changes in splicing patterns. In addition, many Fruitless target neuromodulatory genes, such as

neurotransmitter receptors, ion channels, ion and membrane transporters, and odorant binding proteins are differentially regulated by social context and pheromone signaling. Recent studies showed that social experience and juvenile hormone signaling coregulate *fru* chromatin to modify pheromone responses in olfactory neurons. Interestingly, genes involved in juvenile hormone metabolism are also misregulated in different social contexts and mutant backgrounds. Our results suggest that modulation of neuronal activity and behaviors in response to social experience and pheromone signaling likely arise due to large-scale changes in transcriptional programs for neuromodulators downstream of behavioral switch gene function.

Background

Detection of the social environment through pheromone signaling is critical for animals to recalibrate sex-specific behaviors such as mating and aggression [1-4]. It is thought that changes in social environment can modify the regulation of genes necessary for neuronal homeostasis, physiology, and transmission, ultimately affecting circuit function and behaviors [2, 5, 6]. Previous studies on the effects of early life experience have identified changes in neuroanatomy, synaptic plasticity, neurotransmission, and gene expression. For example, maternal licking and grooming of pups, increases DNA methylation around glucocorticoid receptor gene, leading to long-lasting effects on offspring stress responses and behaviors [7-9]. However, transcriptional cascades driving sensory and social experience-dependent modulation of gene expression, circuit function, and behaviors remain unclear.

Identifying gene regulation cascades by which social signals influence neural and behavioral responses requires a model system with well-defined circuits and genetic regulators with roles in neurophysiology, circuit structure, and behavioral function. Circuitry for courtship behavior in *Drosophila melanogaster* is an excellent experimental system to address this question. In *Drosophila*, male-specific courtship behaviors are governed by a critical transcriptional regulator

49 Fruitless^{M} (Fru^{M}), which is encoded by the male-specific alternative splicing of the *fruitless* (*fru*)
50 gene from the P1 promoter [10, 11]. It is known that Fru^{M} is both necessary and sufficient for
51 male courtship as loss of Fru^{M} in males leads to a loss of male-female courtship [12-14]. Fru^{M} is
52 expressed in approximately 2000 interconnected neurons throughout the peripheral and central
53 nervous system and its expression is required for the development, function, and plasticity of
54 the circuit which drives male-specific behaviors [15]. In particular, social cues such as
55 pheromones can affect courtship behaviors in males [16-21]. Two types of these pheromones,
56 male-specific pheromone *cis*-vaccenyl acetate and non-sex-specific pheromones (such as
57 methyl laurate and palmitoleic acid), activate Fru^{M}-positive olfactory receptor neurons (ORNs)
58 expressing Or67d and Or47b receptors, respectively [18, 20, 21]. These two ORN classes act
59 differently, with Or67d regulating male-male repulsive behaviors and aggression, whereas
60 Or47b driving age and social experience dependent male copulation advantage [4, 20-22].
61 Previous studies have reported that different social contexts, as well as loss of Or47b or Or67d
62 function, alter the regulation of *fru* transcription, particularly the enrichment of active chromatin
63 marks around *fru* promoters [23, 24]. In addition, the expression of *fru^{M}* isoforms in Or47b and
64 Or67d ORNs affects physiological responses to pheromone ligands and courtship behaviors [4,
65 20, 25, 26]. It is likely that changes in social context, pheromone signaling, as well as
66 subsequent changes in *fru* regulation, affect the expression of ion channels as well as
67 neurotransmitter receptors regulating neurophysiology. Indeed, Fru^{M} binding is detected
68 upstream of many ion channels and genes controlling neural development and function in the
69 central brain [27-29]. Even though these studies point to the regulation of neuronal and circuit
70 function by Fru^{M}, very little is known about how it affects the expression of these target genes,
71 or how pheromone signaling and social experience affect transcriptional programs by
72 modulating Fru^{M}.
73 Here we performed antennal RNA-seq to determine transcriptional changes in response to
74 social isolation and mutants in pheromone receptors or Fru^{M}. Our results showed that *fru*

splicing patterns were also modified in pheromone receptor mutants. We also found that transcriptional programs associated with neuromodulation were altered. Many of the Fru^M target neuromodulatory genes were misregulated in the same direction in *fru*^{M} and pheromone receptor mutants. These results uncover a gene regulatory cascade from pheromone receptors to *fru* regulation, which alters neuromodulatory transcriptional programs to ultimately modulate neuronal responses in different social contexts.

Results

Neuronal transcriptional programs are modulated with social isolation and lack of pheromone receptors or Fru^M function.

To identify genes regulated in the peripheral olfactory system by social experience, pheromone signaling, and Fru^M, we utilized RNA-seq from whole antennae of 7-day old wild-type (w^{1118}) males that are either group-housed (w^{1118} *GH*) or single-housed (w^{1118} *SH*), as well as group-housed *Or47b* mutant males (*Or47b*^{1}), *Or67d* mutant males (*Or67d*^{GAL4}), and *fru*^{M} mutant males (*fru*^{LexA}/*fru*$^{4-40}$) (Figure 1A). Each condition included 3 biological replicates except for *fru*^{LexA}/*fru*$^{4-40}$ with only two (Figure 1B). Each sample had mapped reads ranging between 24 and 40 million and hierarchical clustering analysis based on Pearson's correlation between samples showed consistency among replicates within the same genotype (Figure 1B). Principal component analysis (PCA) also showed the expected grouping of the replicates belonging to the same condition, across the first two principal components (PC) accounting for most of the overall variance (32% and 19%) (Figure 1C). We also found that gene expression changes were more similar among *Or67d, Or47b,* and *fru*^{M} mutants, compared to grouped or isolated wild-type male antennae (Figure 1B,C). As expected, expression levels of *Or47b, Or67d*, and male-specific *fru* exon were significantly lower in all replicates for *Or47b*, *Or67d*, and *fru*^{M} mutants, respectively, though the changes of the whole *fru* gene locus cannot be detected (Figure 1D, Figure 4B,

100 Figure 4-figure supplement 1A), validating genotype-specific changes in each condition. In
101 addition, genes known to be absent in adult antennae, such as *amos* [30-32], also showed
102 nearly no expression, whereas housekeeping genes, like *Act5C*, *Gapdh2*, *RpII18*, *fl(2)d*, and
103 *wkd*, showed nearly identical expression across all samples (Figure 1D). These results point to
104 high RNA-seq data quality across sample groups and within biological replicates. The Or47b
105 ORNs were shown to degenerate in 14-day old *Or47b* mutant flies. To test if the transcriptional
106 changes are not due to the decrease of ORN numbers in 7-day old antennal samples, we
107 counted the numbers of Or47b and Or67d ORNs. The total numbers of Or47b or Or67d ORNs
108 were comparable between the control and the *Or* mutants (Figure 1-figure supplement 1).
109 These results suggest that the changes of gene transcriptional level is mainly due to the loss of
110 Or function, rather than the changes of ORN numbers.
111

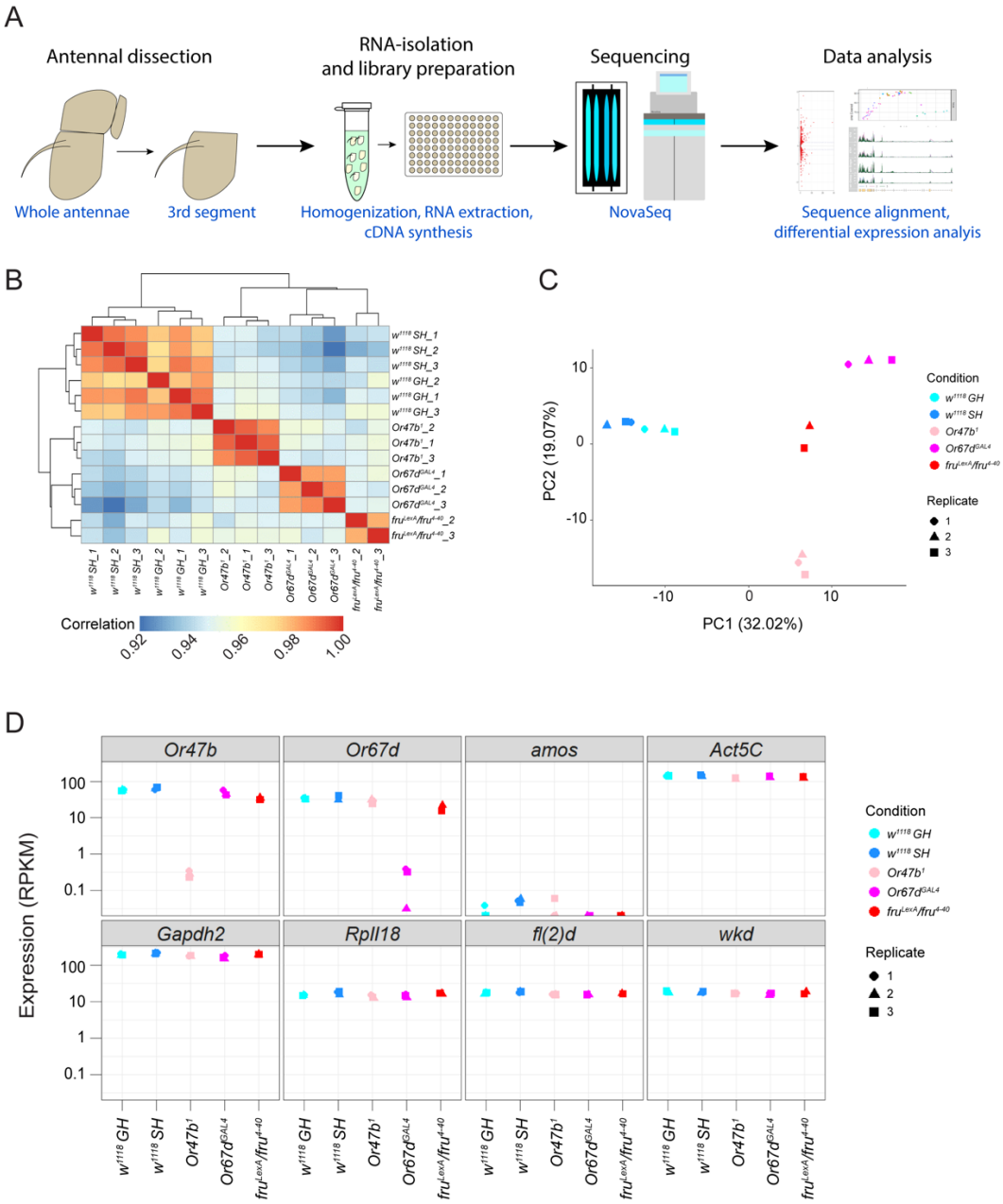


Figure 1. Overview of RNA-seq samples from male antennae. (A) schematic for antennal RNA-seq workflow. **(B-C)** Hierarchical clustering based on Pearson's correlation matrix **(B)** and PCA analysis **(C)** of transcriptional profiles among biological replicates from antennae of wild-type group-housed (*w*^{1118} *GH*), single-housed (*w*^{1118} *SH*), and group-housed *Or47b*^{1}, *Or67d*^{GAL4}, and *fru*^{LexA}/*fru*$^{4-40}$ mutant male flies. **(D)** Transcript levels for several representative negative and positive control genes among all samples.

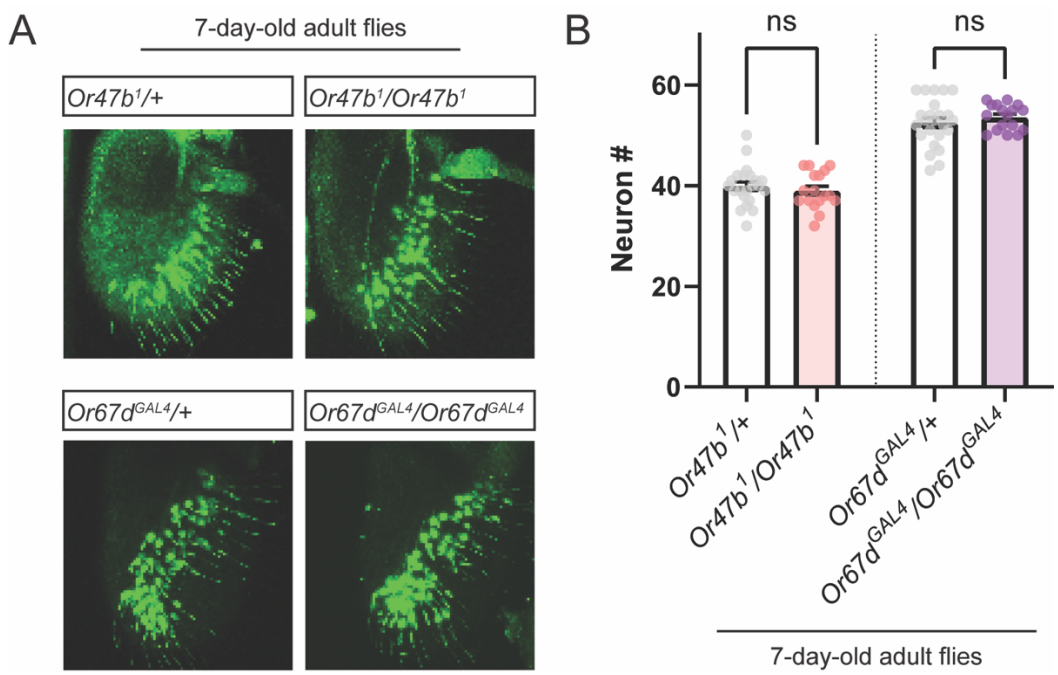


Figure 1-figure supplement 1. ORN numbers of control and mutants. (A) *Or47b-GAL4* driven *UAS-mCD8GFP* expression in 7-day old male antennae from control and *Or47b* mutants (top), and *Or67d*^{GAL4} driven *UAS-mCD8GFP* expression in 7-day old male antennae from control and *Or67d* mutants. **(B)** Statistics of numbers of Or47b and Or67d ORNs in both control and *Or* mutants.

We then ran the differential expression analysis to globally examine the transcriptional changes upon loss of social expression, pheromone sensing, or Fru^{M} function. Compared to group-housed wild-type antennae, social isolation had the least number of significantly altered genes, whereas group-housed *fru*^{M} mutants resulted in the highest number (Figure 2A-C, Supplementary table 1). Pairwise comparisons of group-housed wild types to isolated wild types, and *Or47b/Or67d/fru*^{M} mutants revealed that the genes co-regulated by pheromone receptors and Fru^{M} tended to behave in the same direction in the corresponding mutants (Figure 2A,D), suggesting the shared downstream signaling pathways upon pheromone

135 receptor activation and Fru^M-dependent regulation. The numbers of genes with significant
136 differential expression in the same direction shared by each condition compared to the group-
137 housed wild types are illustrated in a Venn diagram and Upset plot (Figure 2B,C,
138 Supplementary table 2), where genes with overlapping changes in social isolation and *Or47b*,
139 *Or67d*, and *fru^M* mutants are highlighted. Particularly, only one gene, *CG13659*, an ecdysteroid
140 kinase-like domain encoding gene, is consistently changed across all experimental conditions
141 compared to antennae from the group-housed wild-type males (Figure 2B).
142 Hierarchical cluster analysis of differentially expressed genes compared to group-housed wild-
143 type samples showed that the transcriptional changes in *fru^M* and *Or* mutants were most
144 comparable with one another and most dramatically different from the control (Figure 2E).
145 Single-housed wild types were most similar to group-housed wild types (Figure 2E). Cluster
146 analysis identified several genes of behavioral, neuromodulatory, and developmental
147 importance such as *Cytochrome p450 6a20* (*Cyp6a20*)*, serotonin receptor 2A* (*5-HT2A*)*,*
148 *Juvenile hormone esterase* (*Jhe*)*,* and *Dpr-interacting protein alpha* (*DIP-alpha*) (Figure 2E) [33-
149 36]. Among these, antennal expression of *Cyp6a20,* which is downregulated in *Or47b*, *Or67d*,
150 and *fru^M* mutants, was previously shown to mediate effects of social experience on male-male
151 aggression (Figure 2E) [33]. On the other hand, *Cyp4p2,* which is involved in hormone
152 metabolism and insecticide detoxification [37-39], is only misregulated in *Or47b* mutants (Figure
153 2E). In addition to the downregulated genes, we also found some genes encoding ion channels
154 and neurotransmitter receptors that were significantly upregulated (*ppk25* and *GluRIIa*) (Figure
155 2E). The heatmap for gene expression changes revealed gene clusters that were co-regulated
156 by pheromone receptors and Fru^M, in addition to gene clusters that were uniquely regulated by
157 each OR and Fru^M; this again highlights that the co-regulated genes tend to change in the same
158 direction in pheromone receptor and *fru^M* mutants.
159

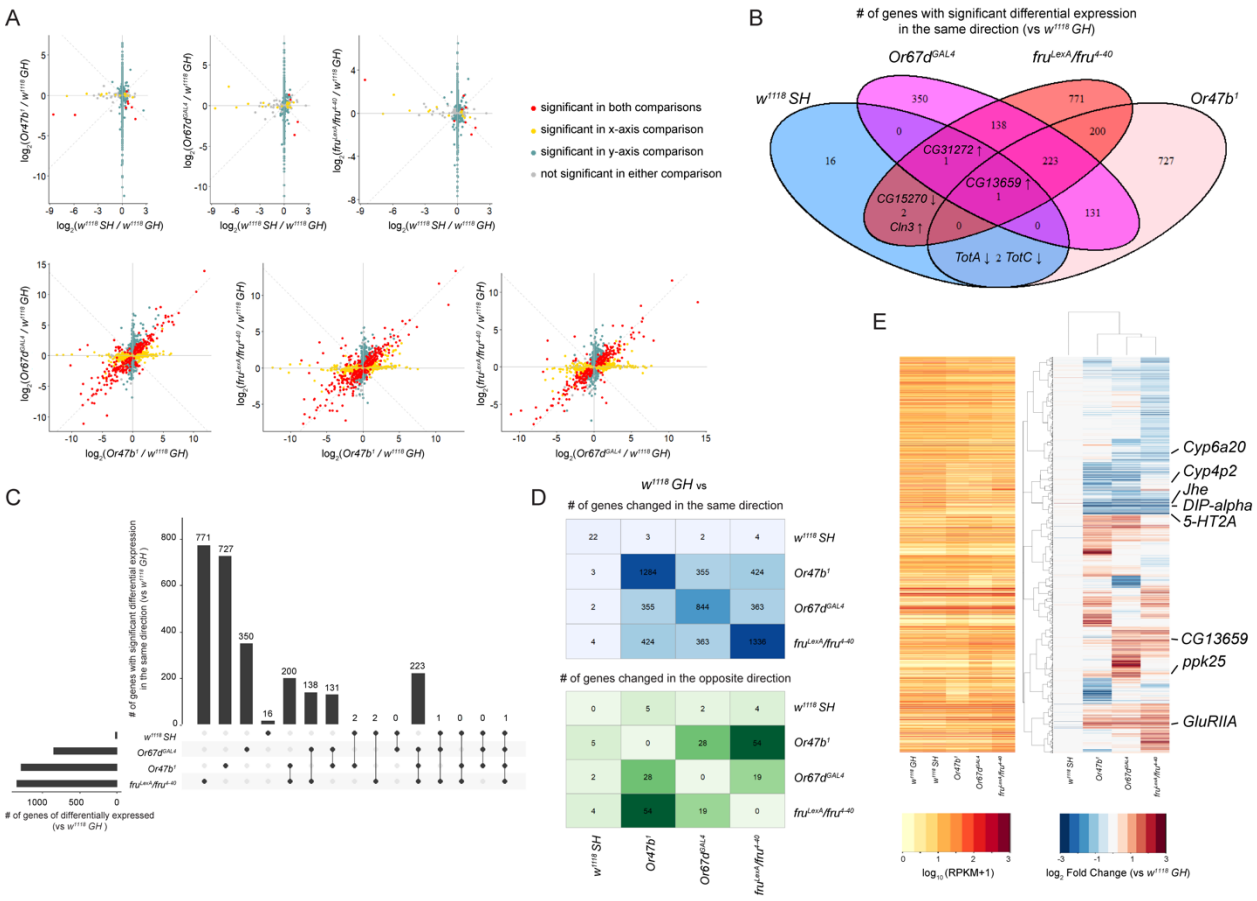


Figure 2. Differentially expressed genes in response to loss of social experience, pheromone receptors, or *fru*^{M}. (A) Scatter plot showing the genes that are differentially regulated among social isolation, and mutants in pheromone receptors and *fru*^{M}. Significance is defined by adjusted p-value below 0.01 after applying Bonferroni correction with n = 2. **(B-C)** Venn diagram **(B)** and UpSet plot **(C)** comparing differentially expressed genes shared across experimental conditions (only genes changed in the same direction). **(D)** Numbers of differentially expressed genes with the same (top) and the opposite (bottom) direction in pairwise comparison of experimental conditions versus group-housed wild-type samples. In (B-D), significance is defined by adjusted p-value below 0.01 after applying Bonferroni correction with n = 4. **(E)** Hierarchically clustered heatmaps showing $\log_2$ fold change compared to group-housed wild-type antennae across all experimental conditions (right) and average mRNA levels (reads per kilobase of transcript, per million mapped reads, RPKM) of replicates within each

173 condition ordered in the same way as $\log_2$ fold change (left). Only 2999 genes with at least one

174 significant (adjusted p-value below 0.01) change between an experimental condition versus

175 group-housed wild types are shown.

176

Gene ontology terms for differentially expressed genes in response to lack of social and pheromone signaling highlight neuromodulators

Previous work has demonstrated that social experience, pheromone signaling, and Fru^M activity can regulate the responsiveness of pheromone sensing ORNs to modify neuronal function and sex specific behaviors [4, 18, 20, 21, 33, 40]. To functionally understand system-level changes in gene expression with social isolation, lack of pheromone signaling, and *fru*^{M} mutants, we next investigated gene ontology (GO) terms using GOrilla for the list of differentially expressed genes in each experimental condition in pairwise comparisons with group-housed wild types [41, 42] (Figure 3, Supplementary table 3). Many GO terms of molecular function and biological process were commonly affected across multiple experimental groups, suggesting the converging downstream molecular events in response to social experience and pheromone sensing mediated by Fru^M activity (Figure 3). Strikingly, the genes with the altered expression tended to be localized on the cell membrane (Figure 3, GO: cellular component) and have functions in ion transport across membrane (Figure 3, GO: molecular function), and appeared to be involved in the process of detecting and responding to olfactory stimuli (Figure 3, GO: biological process). This supports previous studies in providing a general mechanism for social experience, pheromone receptor signaling, and Fru^M-dependent regulation of pheromone responsiveness of Or47b ORNs [4, 24, 25]. Furthermore, genes with oxidoreductase activity also had overlapping alterations across *Or47b*, *Or67d,* and *fru*^{M} mutants, and many of these appeared to contribute to insect hormone metabolism (Figure 3, GO: molecular function). Interestingly, previous studies reported that juvenile hormone signaling works together with social experience in olfactory receptor neurons to modulate chromatin around *fru* locus [4, 24]. Our RNA-seq results also add an additional layer of complexity to hormone-social experience interactions, as social experience and pheromone signaling affects the levels of certain hormones by modifying hormone metabolism dynamics. In summary, social isolation, disrupted pheromone receptor

signaling, and lack of Fru^M function in peripheral olfactory sensory neurons affect the expression of many genes with roles in diverse aspects of neurophysiology, including neuronal responsiveness, ion transmembrane transport, and beyond.

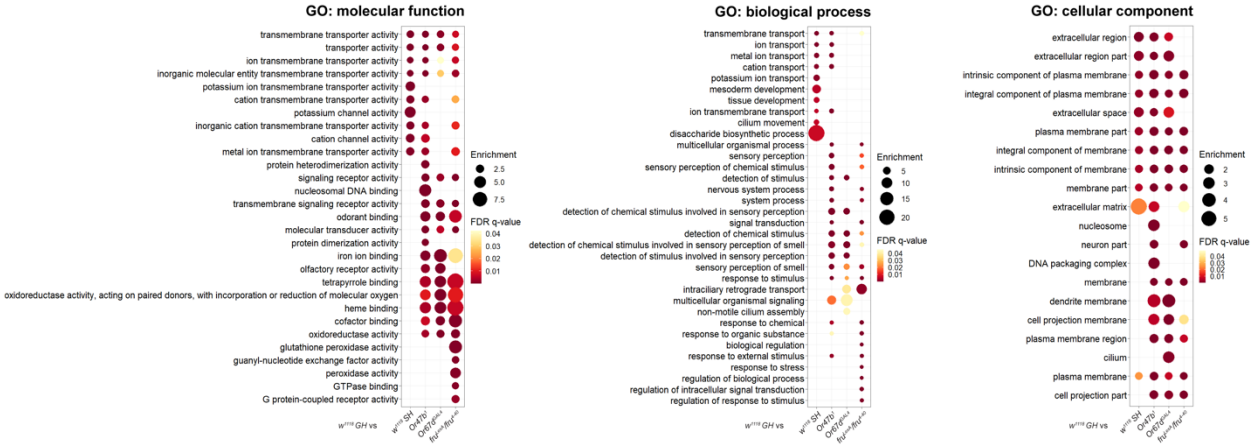


Figure 3. Top enriched gene ontology (GO) terms for differentially expressed genes in response to social experience, pheromone signaling, and Fru^M function. The union set of top 10 most significantly enriched GO terms with FDR q-value below 0.05 of the differentially expressed genes in each experimental condition is shown. Enriched GO terms were generated by the single ranked gene list with the most significantly changed genes at the top via GOrilla.

Loss of pheromone signaling alters *fruitless* splicing patterns and *doublesex* expression

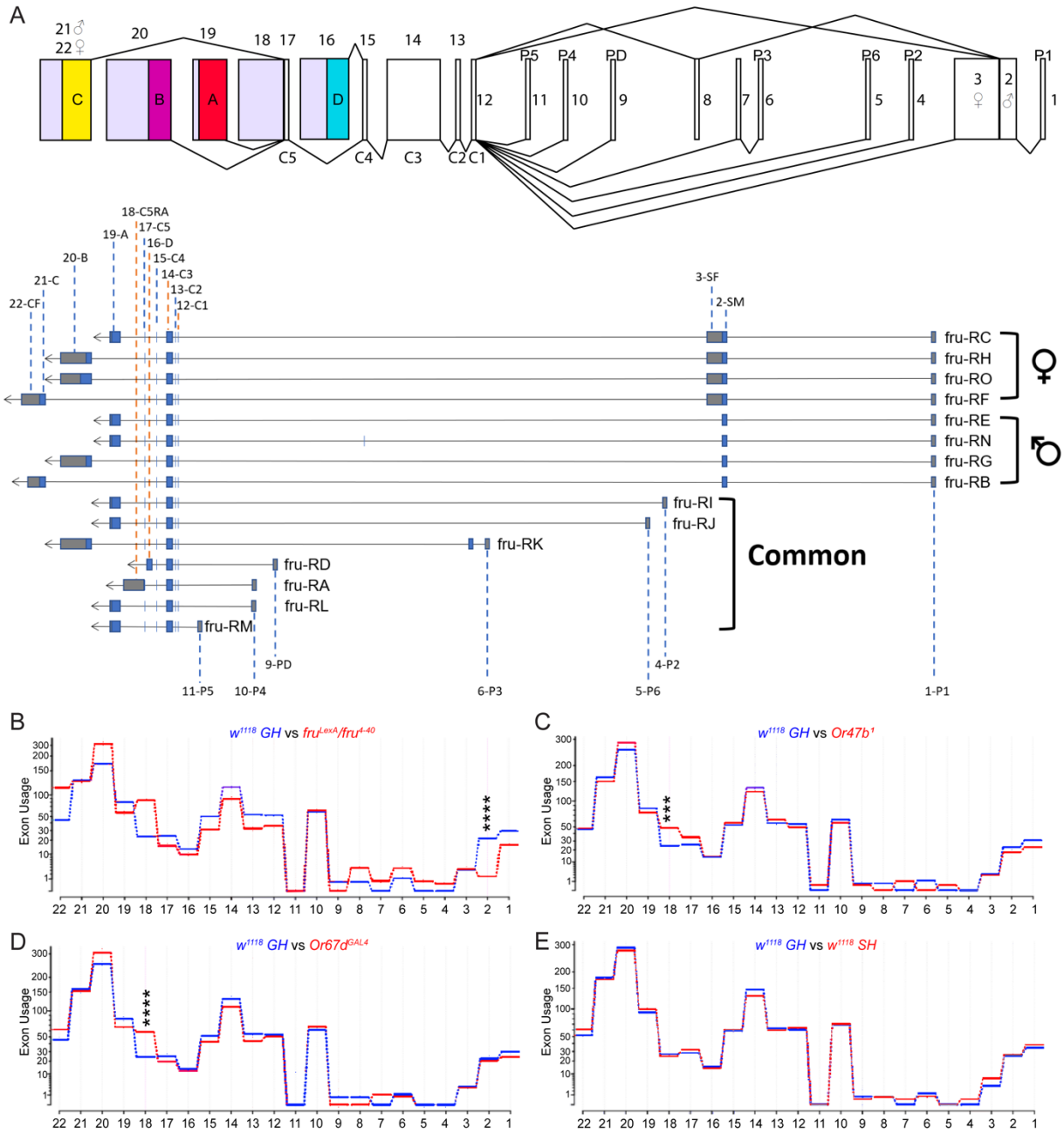
Fruitless locus generates multiple alternative splice isoforms for RNAs transcribed from 7 promoters (P1-P6, PD). The transcripts from *fru P1* are alternatively spliced between males and females, where the male, but not the female, isoform (fru^M) encodes functional proteins (10,11). The expression of fru^M in males and the absence of functional fru^F transcripts in females help define male and female-specific neuronal pathways as well as the cell-specific expression patterns of genes regulated by Fru^M. Promoter *fru P2* through *fru P6* produce common isoforms in both males and females that also affect sex-specific activity in courtship circuits of both sexes [43] (Figure 4A). Fru^M itself has multiple splicing isoforms that vary in the 3' end of the mRNA (fru^{MA}, fru^{MB}, fru^{MC}), which encode proteins with variable zinc finger DNA binding domain of Fru^M transcription factor [27, 29, 43]. These regulate different aspects of the circuit controlling courtship behaviors, with Fru^{MC} and Fru^{MB} having the highest overlap behaviorally, and Fru^{MA} having little to no effect on courtship [27].

We previously showed that social experience and signaling from Or47b and Or67d pheromone receptors alter open chromatin marks around *fru P1* promoter in the male antennae [24]. Interestingly, examination of total transcript levels for the entire *fru* gene locus showed little to no difference across experimental conditions (Figure 1D). These small changes in total transcript levels, despite dramatic changes in open chromatin marks in wild-type SH, and mutants in *Or47b*, *Or67d,* and fru^M, prompted us to look at other aspects of gene regulation. It is known that changes in chromatin regulate many aspects of transcription such as transcriptional initiation, elongation and alternative splicing [44, 45]. The effects of chromatin on splicing are thought to occur especially because chromatin state alters the speed of RNA Polymerase II (RNAPII), which can lead to splicing mistakes like intron retention or exon skipping [45].

237 Given the functional differences in the *fru*^{M} isoforms, we predicted that chromatin changes
238 caused by social experience and pheromone receptor signaling could alter *fru* splicing. To
239 explore this, we mapped reads from all experimental conditions to *fru* genomic locus and
240 investigated exon usage levels using DEXseq [46]. In general, transcript reads from *fru* locus
241 appear noisier in experimental conditions compared to group-housed wild-type male antennae,
242 with variations in the expression of coding and non-coding sequences (Figure 4B-E). In *Or47b*
243 mutants, there is a small decrease in *fru P1* promoter (region 1, see methods – statistical
244 analysis) and male-specific exon (region 2, see methods – statistical analysis) levels (Figure
245 4C). *Or67d* mutants show a small decrease in *fru P1* promoter levels (see methods – statistical
246 analysis) and male-specific exon (region 2; see methods – statistical analysis) (Figure 4D). The
247 largest change in male-specific exon levels is seen in *fru*^{LexA}/*fru*$^{4-40}$ allele (Figure 4B), which has
248 a *LexA* transgene insertion disrupting the male-specific exon and a 70-Kb deletion from the *P1*
249 promoter [47]. *fru P1* promoter and the male-specific exon are unaltered in socially isolated
250 antennae, yet there is a small increase in the female-specific exon (region 3, see methods –
251 statistical analysis) (Figure 4E). In addition to the first 3 exons, a non-coding sequence (region
252 18) (Figure 4A), which is a only present in *fru-RA* transcript (43), strikingly increases in *Or67d,*
253 *Or47b*, and *fru*^{M} mutants (Figure 4B-D). This transcript encodes a Fru protein that lacks these
254 zinc finger domains but retains BTB/PDZ protein-protein interaction domain (Figure 4A). It is
255 possible that this isoform can interfere with the transcriptional functions of Fru^{M} proteins by
256 binding and titrating out their interaction partners such as other transcription factors, chromatin
257 modulators, and basal transcriptional machinery [48-51]. These results suggest social and
258 pheromonal cues alter *fru* exon usage, likely indicating changes in *fru* splicing patterns.
259 Another sex determination transcription factor known to regulate sex specific behaviors is
260 *doublesex (dsx)* [52-60]. *dsx* expression in the antenna is restricted to non-neuronal cells [58].
261 We found that the expression of *dsx* in antenna is significantly increased in *Or* and *fru*^{M} mutants,
262 albeit the increase is much more pronounced in *Or67d* and *fru*^{M} mutants (Figure 4-figure

supplement 1B). Socially isolation did not alter the expression of *dsx* in antennae (Figure 4-figure supplement 1B,D). These results suggest that the expression of *dsx* in the antennae is repressed by *Or47b, Or67d* and *fru*^{M} function.

In conclusion, our results suggest that the expression of two critical transcription factors, Fru and Dsx, that regulate sex-specific behaviors are modulated by pheromone signaling.



270 **Figure 4. Olfactory stimuli regulate exon usage across the whole *fru* genomic locus. (A)**
271 Top: schematic of Fruitless: 1-22 denote each examined site. P1-P6 and PD denote promoters.
272 C1-C5 denote common exons, while A-D denote alternatively spliced 3' DNA binding-encoding
273 domains. Bottom: structure of *fru* transcripts with the exon names and numbering used in the
274 schematic above. **(B-E)** Examination of the usage of various exons in Fruitless to determine
275 distinct changes in fru^{M} transcripts using DEXSeq, showing exon-by-exon comparison of w^{1118}
276 *GH* vs fru^{LexA}/fru^{4-40} **(B)**, $Or47b^{1}$ **(C)**, $Or67d^{GAL4}$ **(D)**, and w^{1118} *SH* **(E)**. Adjusted p-value was
277 directly performed via DEXSeq (see methods – statistical analysis). ***, p.adjust<0.001; ****,
278 p.adjust<0.0001. Region 2, w^{1118} *GH* vs fru^{LexA}/fru^{4-40}, p.adjust=1.25×10^{-10}; region 18 (3'UTR),
279 w^{1118} *GH* vs $Or47b^{1}$, p.adjust=8.17×10^{-4}; region 18 (3'UTR), w^{1118} *GH* vs $Or67d^{GAL4}$,
280 p.adjust=2.00×10^{-7}.
281

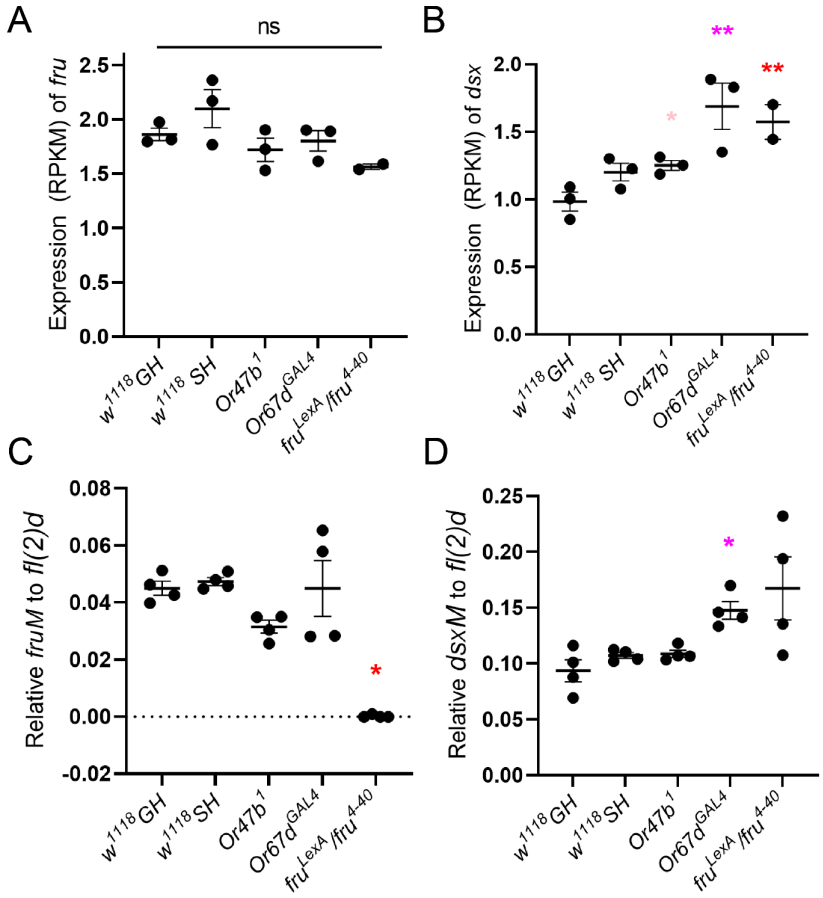


Figure 4-figure supplement 1. Validation of *fru* and *dsx* male specific exon expression in male antennae of WT and mutants. (A-B) RPKM of *fru* and *dsx.* Adjusted p-value was directly performed via DESeq2. **(C-D)** Quantitative RT-PCR validation of *fru* and *dsx* male specific exon expression. **(C)** The expression of fru^M was not detected in *fru* mutants and did not show significant difference in other *Or* mutants or SH WT. **(D)** The dsx^M expression showed significant increase in *Or67d* mutant male antennae.

291 **Bimodal regulation of genes modulating neurophysiology and**

292 **neurotransmission by Fru^M and pheromone receptor signaling**

293 Previous studies have shown that pheromone receptor signaling and social experience-
294 dependent regulation of chromatin and RNAPII enrichment around *fru P1* promoter can
295 ultimately scale and fine-tune behavioral responses to social environment [4, 24]. Additionally,
296 previous reports on the genome-wide binding profiles for three Fru^M isoforms in the central brain
297 revealed isoform-specific differences in target genes that regulate neuronal development and
298 function [27, 52]. Fru^M motifs are enriched among regulatory elements that are open in the
299 female but closed in the male, suggesting Fru^M functions as possible repressive transcription
300 factor [61]. Functional differences of Fru^M isoforms also influence ORN responses to their
301 pheromone ligands [25]. Thus, chromatin-based modulation of *fru* levels and splicing with social
302 experience and pheromone signaling can provide a quick way to modulate neuronal physiology
303 and synaptic communication by modifying gene expression programs. Yet, the effect of social
304 experience and pheromone receptor signaling on gene expression programs or the mode of
305 gene regulation by Fru^M (as a transcriptional activator, repressor, or both) remains unknown.
306 As discussed previously, gene ontology analysis of these differentially expressed genes implies
307 that many genes involved in neuromodulation are regulated by social context, pheromone
308 receptor signaling, and Fru^M function. To further investigate this, we specifically focused on
309 genes associated with ion channel activity and/or neurotransmitter regulation (Figure 5A,B;
310 Figure 6A,B). We clustered these genes based on their log_2 fold change in transcript levels
311 compared to group-housed wild types in each experimental condition, while also showing their
312 corresponding expression levels in the antennae (RPKM, reads per kilobase of transcript, per
313 million mapped reads) (Figure 5A,B; Figure 6A,B). We found many ion channel and/or
314 neurotransmitter receptor-encoding genes showed up/down-regulation in response to social
315 isolation, and loss of Or47b, Or67d, or Fru^M function (Figure 5A,B; Figure 6A,B). Within ion

316 channels, two subclasses stood out. These are the Degenerin/Epithelial Sodium Channel
317 (DEG/ENaC) proteins known as pickpockets (ppks) and inward-rectifying potassium channels
318 Irks. Additional genes also include those encoding calcium channels, for example, *Piezo*, *TrpA1*,
319 and *cacophony* (*cac)* (Figure 5A,B).
320
321 ***ppk gene family***
322 Recent reports pointed to the function of DEG/ENaC channels known as *pickpocket* family of
323 sodium channels that act in Or47b and Or67d ORNs to regulate responses to their ligands [25].
324 Fru^M binding motifs have been identified around many of these *ppk* family members, such as
325 *ppk*, *ppk5*, *ppk6, ppk15, ppk19*, *ppk23*, *ppk25,* and *ppk30* [27, 29, 62]. Both *ppk23* and *ppk25*
326 have been identified as necessary for modulating responses of Or47b ORNs through Fru^{MB} and
327 Fru^{MC} activity, respectively, with Fru^{MB} having an antagonistic effect on physiology in Or67d
328 ORNs [25, 26]. We therefore first examined the expression levels of *ppk* genes in the antennae.
329 In group-housed wild-type antennae, *ppks* show generally low expression, with *ppk5* displaying
330 the highest levels (Figure 5C). Many *ppk* genes, including *ppk23,* seem not to be expressed in
331 antenna. Analysis of recent single-cell RNA-seq data from ORNs [63] revealing ORN class-
332 specific expression of *ppk*s also agree with this pattern. However, a few *ppks* known to be
333 expressed in several ORN classes are not detectable in this dataset, possibly due to the
334 limitations of single-cell RNA-seq in detecting low-abundance genes (Figure 5-figure
335 supplement 1A). Even though there is no effect on the expression of any *ppk* family members in
336 socially isolated male antennae, many *ppk* genes are differentially regulated in fru^M mutants, in
337 agreement with the existing Fru^M binding sites at their promoters. For example*, ppk6 and ppk25*
338 are upregulated in fru^M mutants whereas *ppk5*,*7*,*13*,*14*,*15*,*19* are downregulated. Many of these
339 genes also show correlated changes in *Or47b* and/or *Or67d* mutants (*ppk13*,*14*,*15*,*19*,*25*). *ppk6*
340 is strikingly upregulated in both fru^M and *Or67d* mutants, whereas *ppk7* is downregulated in both
341 *Or47b* and fru^M mutants (Figure 5C'). Of note is the significant increase in *ppk25* expression,

342 especially in *Or67d* mutants. *ppk25* is expressed in Or47b and Ir84 ORNs, but not Or67d ORNs,
343 and has recently been shown to be downstream of Or47b and Ir84a activity altering their
344 neuronal responses [26, 64, 65] (Figure 5C). The changes in *ppk25* levels were also validated
345 through quantitative RT-PCR (Figure 5-figure supplement 1C-E). The bimodal changes in *ppk*
346 transcripts in fru^{M} mutants suggest Fru^{M} can act as both a repressor and an activator of *ppk*
347 gene regulation. Furthermore, given that pheromone receptor function regulates Fru, the
348 patterns of *ppk* gene misregulation in *Or47b* and *Or67d* mutants are likely dependent on
349 changes in Fru^{M} activity downstream of pheromone receptor signaling.
350
351 ***Irk gene family***
352 Irk gene family encodes 3 inwardly rectifying potassium channels (Irk1-3) with binding motifs for
353 Fru^{MA} identified upstream of *Irk2* and binding of both Fru^{MA} and Fru^{MC} found around *Irk3* [27, 29,
354 62]. Three *Irk* genes are expressed in varying levels in the antennae with *Irk1* having the lowest
355 expression and *Irk2* having the highest expression (Figure 5D). We found that *Irk1* is
356 upregulated in *Or47b* mutants, whereas *Irk2* shows the trend towards upregulation in response
357 to social isolation (Figure 5D').
358 These results suggest that changes in the transcript levels of Fru^{M}-regulated sodium and
359 potassium channels with social isolation and in pheromone receptor mutants may contribute to
360 changes in neuronal responses and behaviors.
361

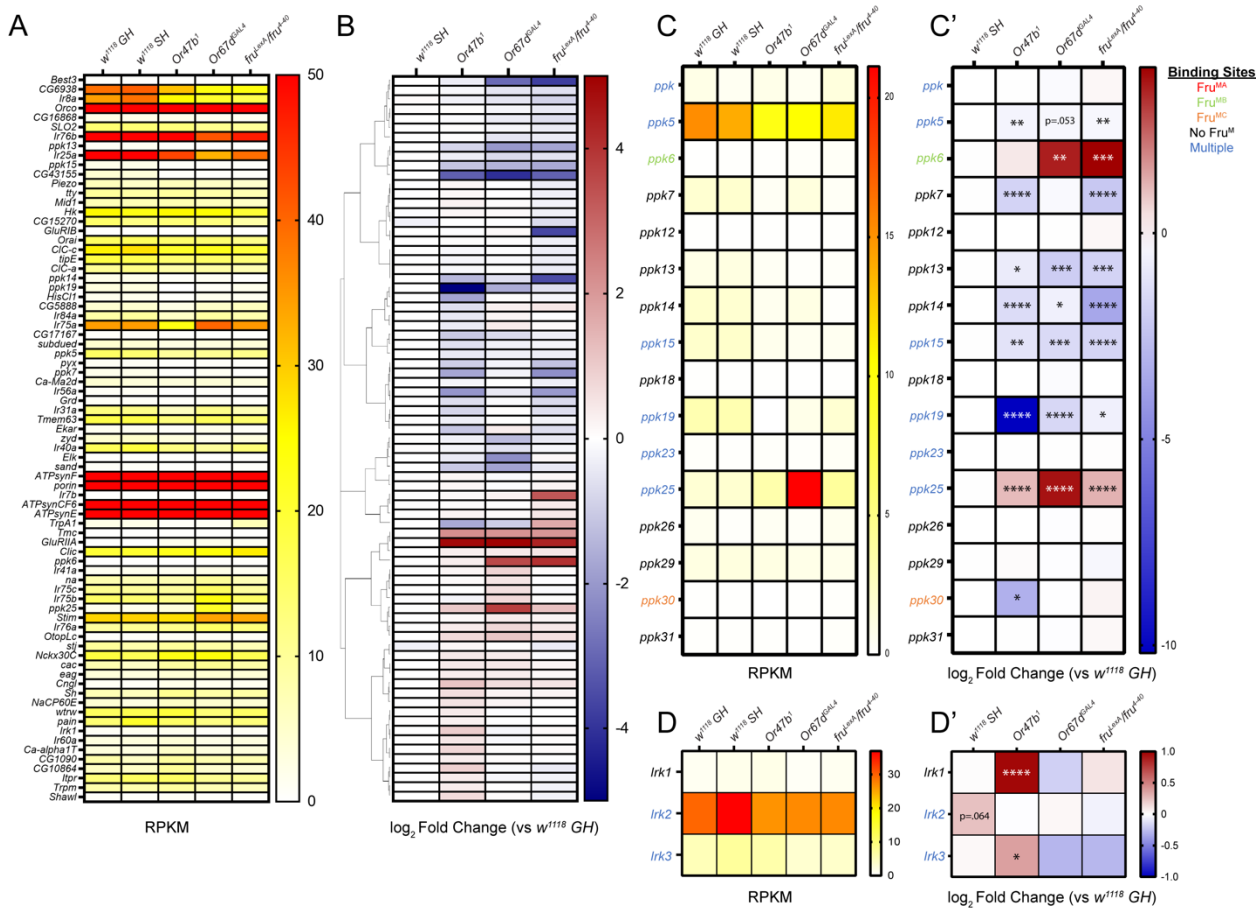


Figure 5. Differentially expressed ion channel-encoding genes in response to social isolation and loss of pheromone receptors or *fru*^{M}. (A-B) Examination of GO term: 0005216 (ion channel activity) shows significant changes in various ion channel subclasses. Hierarchically clustered heatmaps showing $\log_2$ fold change compared to group-housed wild-type antennae across all experimental conditions **(B)** and average mRNA levels (RPKM) of replicates within each condition ordered in the same way as $\log_2$ fold change **(A)**. Genes with adjusted p-value above 0.01 were filtered out in each experimental condition. **(C-C')** RPKM **(C)** and $\log_2$ fold change **(C')** for *pickpocket* (*ppk*) gene family. **(D-D')** RPKM **(D)** and $\log_2$ fold change **(D')** for *inwardly rectifying potassium channel* (*Irk*) gene family. Adjusted p-value was directly performed via DESeq2. *, p.adjust<0.05; **, p.adjust<0.01; ***, p.adjust<0.001; ****, p.adjust<0.0001. Fru^{M} binding information is listed in Supplementary table 4.

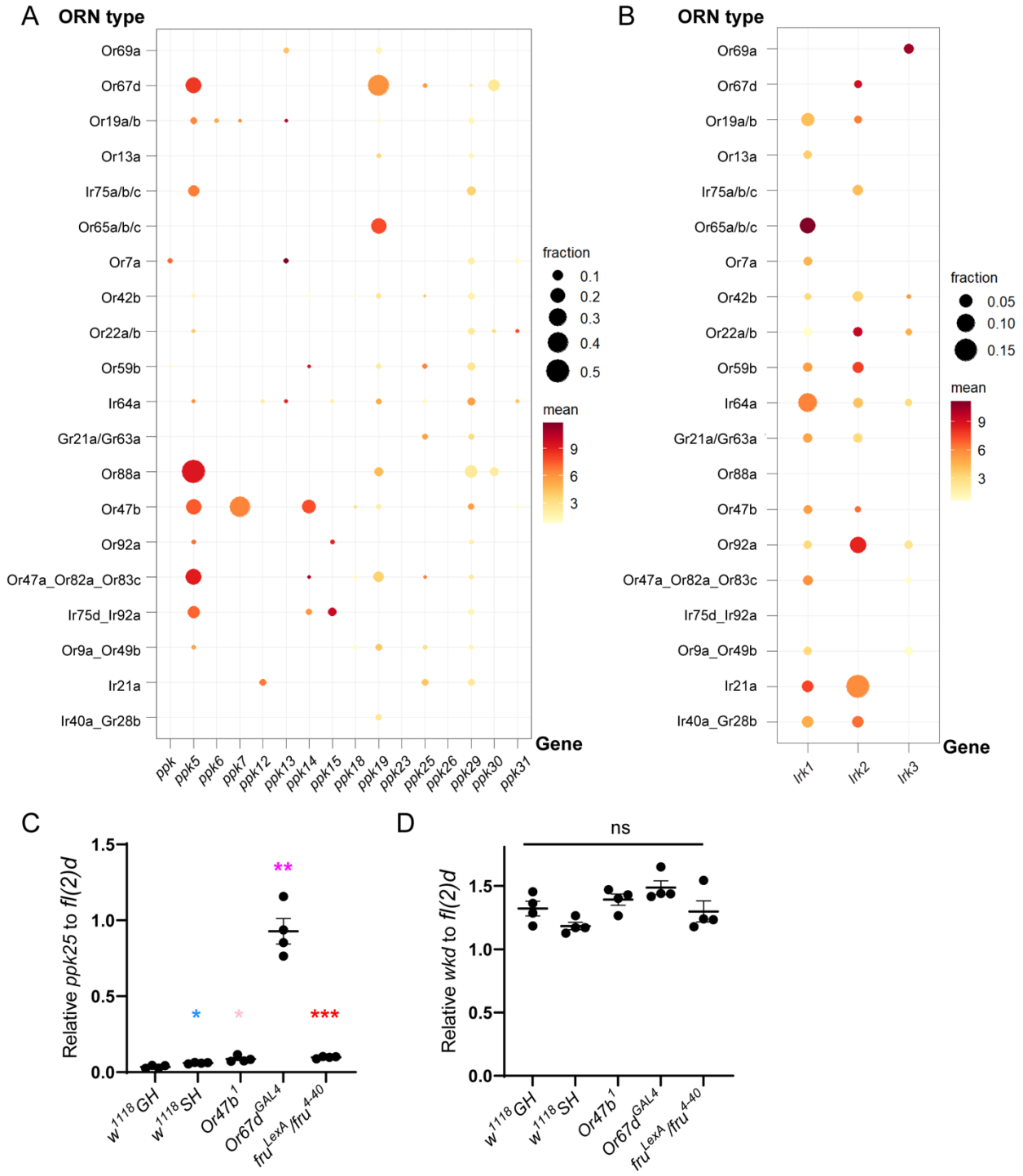


Figure 5-figure supplement 1. Validation of *ppk* expression across ORN classes and in mutants. (A-B) ORN class-specific expression of *ppk* and *Irk* family genes based on single-cell RNA-seq datasets from the adult ORNs [66]**.** Size of each circle indicates the fraction of positive cells ($\log_2$(CPM+1)>0.5) and color intensity indicates the mean expression ($\log_2$(CPM+1)) of all

380 positive cells. **(C-D)** Quantitative RT-PCR validation of *ppk25* expression (C) and a negative
381 control gene *wkd* expression (D) from antennae of grouped and socially isolated wild types,
382 *Or47b* mutants, *Or67d* mutants, and fru^{M} mutants normalized to *fl(2)d*. *fl(2)d* and *wkd* were
383 selected based on their near-identical expression level across all conditions from the RNA-seq
384 results and thus used as loading and negative control genes.
385

386 ***Regulators of Neurotransmission***

387 To ask if social experience, pheromone signaling, and Fru^M function regulate genes involved in
388 neurotransmission, we next examined the expression of neurotransmitter receptors,
389 transporters, and enzymes for neurotransmitter metabolism. ORNs in the antennae as well as
390 their projection neuron targets around the antennal lobes are mostly cholinergic [67]. In the
391 antennal lobe it has been shown that local interneurons, which include serotonergic,
392 GABAergic, and glutamatergic interneurons, provide cross talk between synaptic partners in the
393 antennal lobe glomeruli [67, 68]. These neurons form connections with both presynaptic ORNS
394 and their postsynaptic partner projection neurons for modulation of neuronal response across
395 glomeruli [67, 69, 70]. These connections are required for fine tuning of signaling at synapses
396 as a way of rapid modulation of neuronal function [34, 69-77]. We found a high expression of
397 choline acetyltransferase (*ChAT*) that catalyzes acetylcholine biosynthesis and *VAChT* that
398 packages acetylcholine into synaptic vesicles, coinciding with their reported cholinergic roles in
399 ORNs. Moreover, we also found relatively high expression of several genes encoding receptors
400 of various neurotransmitters, such as choline, serotonin (5-HT), GABA, and glutamate (Figure
401 6C-F'). Many of these genes, such as *nAChRalpha4*/*5*, *5-HT2A*, *5-HT7*, *GABA-B-R2*, and
402 *GluRIIA*, have previously been found to regulate courtship behavior in flies through signaling in
403 the antennal lobe [73, 78-80]. Interestingly, *GABA-B-R2* was shown to be specifically involved in
404 presynaptic gain control of Or47b ORNs [81]. Additionally, single-cell RNA-seq data shows both
405 broadly expressed neurotransmitter genes like *GluRIIB* and 5-*HT2B*, while others are specific to
406 a subset of ORN classes [66] (Figure 6-figure supplement 1). Overall, many of the genes
407 encoding neurotransmitter receptors show expression changes in different experimental
408 conditions (Figure 6B).

409 To focus on genes related to specific neurotransmitters, we didn't observe any significant
410 changes in response to social isolation, except for a few genes, like *dmGlut,* which is
411 upregulated compared to the group-housed wild types (Figure 6D,D'). We again found that loss

412 of Fru^M function led to bimodal effects on gene expression (Figure 6C-F'). Indeed, many of
413 these genes have known Fru^M binding to their promoters, including receptors
414 *nAChRalpha1*/*3*/*4*/*5,* *GluRIIA*, *GluClalpha*, *5-HT1A*, *5-HT1B*, *5-HT2A*, *5-HT7*, and
415 transporters/regulators such as *VAChT, ChAT*, and *Gat* [27, 29, 62]. Some of these genes
416 display correlated changes between pheromone receptor mutants and fru^M mutants, like
417 *GluRIIA*, *dmGlut*, and *5-HT2A*, suggesting that the effects of pheromone signaling on
418 neurotransmission can act via their influences on *fru* regulation (Figure 6D-E'). The changes in
419 *5-HT2A* were also validated through qRT-PCR (Figure 6-figure supplement 1C). In the antenna,
420 *5-HT2A-GAL4* and *dmGlut-GAL4* expression is observed in a subset of ORNs (Figure 6G-H').
421 Interestingly, Or47b and Or67d ORNs do not express *5-HT2A* reporter (Figure 6G). In
422 agreement with a decrease in *5-HT2A* transcript levels in the RNA-seq and RT-PCR
423 experiments, *5-HT2A* reporter expression was significantly decreased in *Or47b* mutant
424 antennae (Figure 6G-G'). On the other hand, *dmGlut* expression in the antennae was
425 upregulated in all conditions compared to group housed male antennae, generally in agreement
426 with the qRT-PCR validation results (Figure 6D,D', Figure 6-figure supplement 1D). We also
427 used a *dmGlut-GAL4* to visualize the *dmGlut* expression in vivo, and detected the signal in a
428 subset of non-neuronal cells in the antennae, though we only observed the statistically
429 significant increase of the *dmGlut* reporter expression in fru^M mutants (Figure 6H,H'). Evident
430 changes are also observed in some genes not known to be Fru^M targets, for example, *GluRIB*
431 which shows downregulation only in fru^M mutants, and *5-HT2B* which shows upregulation in
432 *Or47b* and fru^M mutants (Figure 6D-E'). These may reflect effects of pheromone receptor
433 signaling independent of Fru^M function or indirect effects of Fru^M activity. To summarize, the
434 systems-level changes in expression of genes involved in neurotransmission and
435 neurophysiology with social experience and pheromone receptor signaling can modulate ORN
436 responses. In addition, these effects on gene expression with social signals can occur either in

437 a Fru^M-dependent manner or independently of Fru^M in response to other gene regulatory

438 pathways activated by pheromone receptor signaling.

439

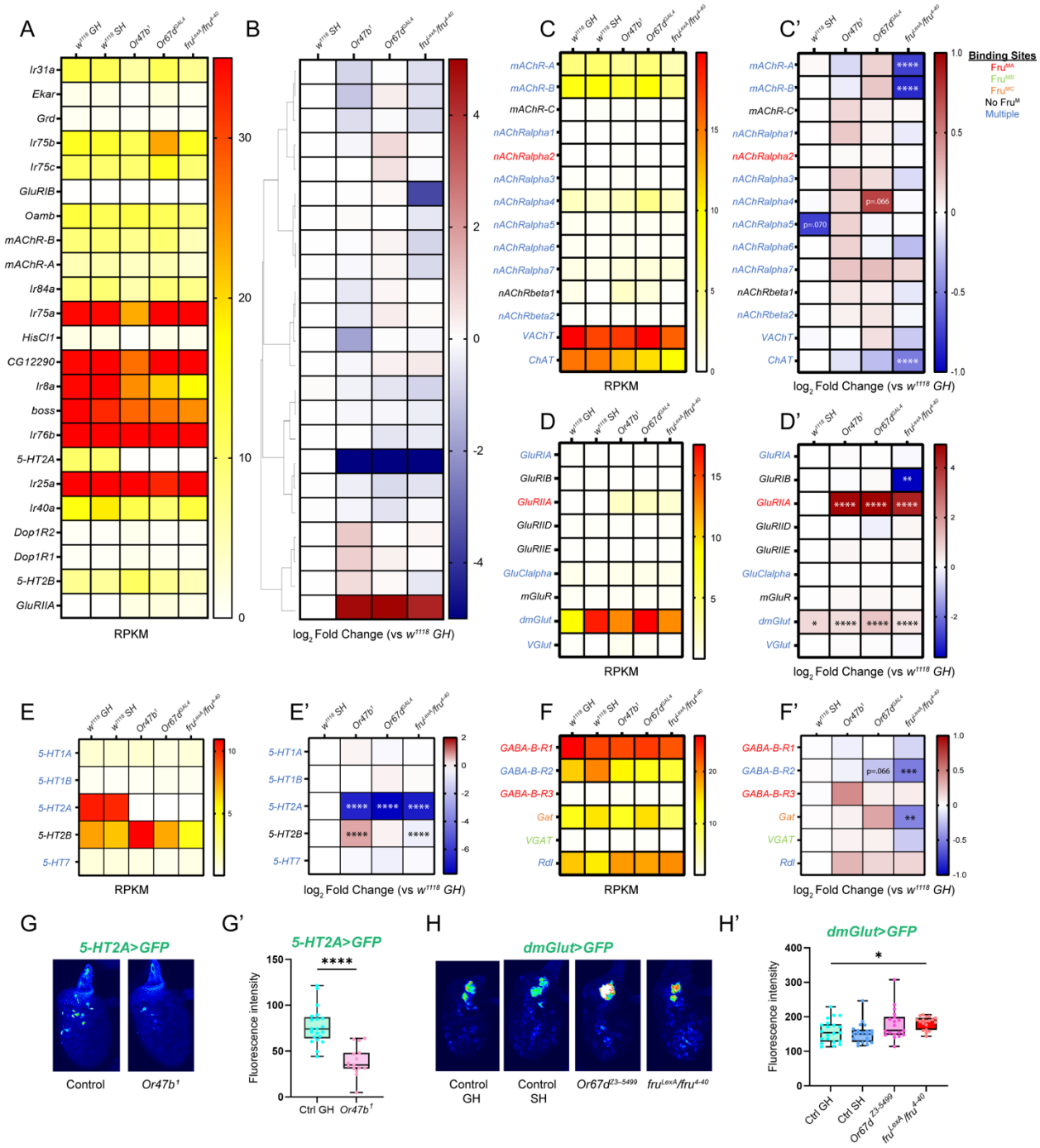


Figure 6. Differentially expressed neurotransmitter receptor and transporter-encoding genes in response to social isolation and loss of pheromone receptors or *fru*^{M}. (A-B) Examination of GO term: 0030594 (neurotransmitter receptor activity) shows significant changes in various neurotransmitter activity-associated subclasses. Hierarchically clustered heatmaps showing $\log_2$ fold change compared to group-housed wild-type antennae across all

experimental conditions **(B)** and average mRNA levels (RPKM) of replicates within each condition ordered in the same way as log_2 fold change **(A)**. Genes with adjusted p-value above 0.01 were filtered out in each experimental condition. **(C-C')** RPKM **(C)** and log_2 fold change **(C')** for acetylcholine-associated genes. **(D-D')** RPKM **(D)** and log_2 fold change **(D')** for glutamate-associated genes. **(E-E')** RPKM **(E)** and log_2 fold change **(E')** for serotonin-associated genes. **(F-F')** RPKM **(F)** and log_2 fold change **(F')** for GABA-associated genes. Adjusted p-value was directly performed via DESeq2. *, p.adjust<0.05; **, p.adjust<0.01; ***, p.adjust<0.001; ****, p.adjust<0.0001. Fru^M binding information is listed in Supplementary table 4. **(G, H)** Confocal images of antenna from WT GH, WT SH, *Or47b*^{1}, *Or67d*$^{Z3-5499}$, and *fru*$^{LexA/4-40}$ mutants expressing *5HT2A-GAL4* driven *40XUAS-mCD8GFP* (**G)** and *dmGlut-GAL4* driven *UAS-mCD8GFP* **(H)**. Quantification of fluorescence in images G **(G')**, and H **(H')**. **(G-G')** n=15-21. **(H-H')** n=19-21.

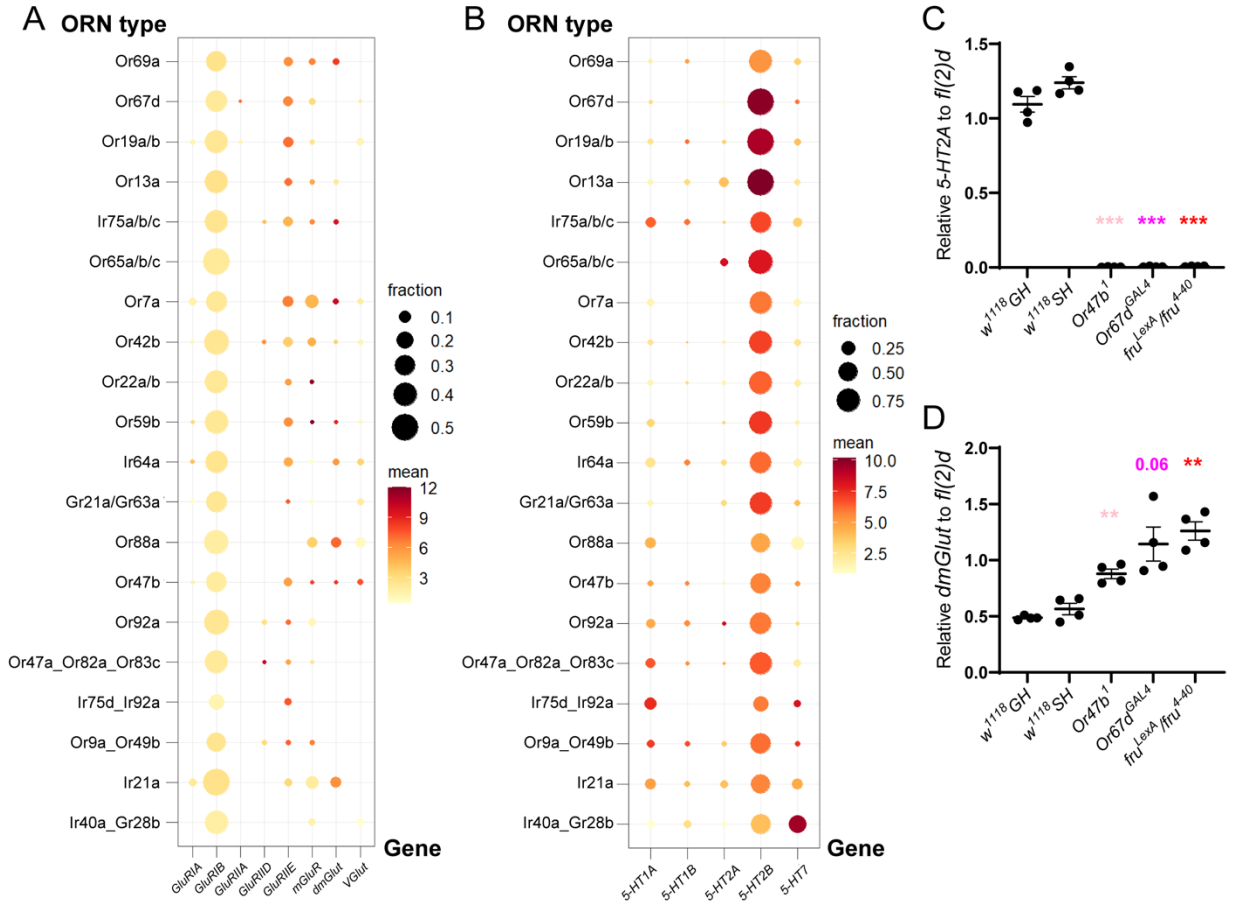
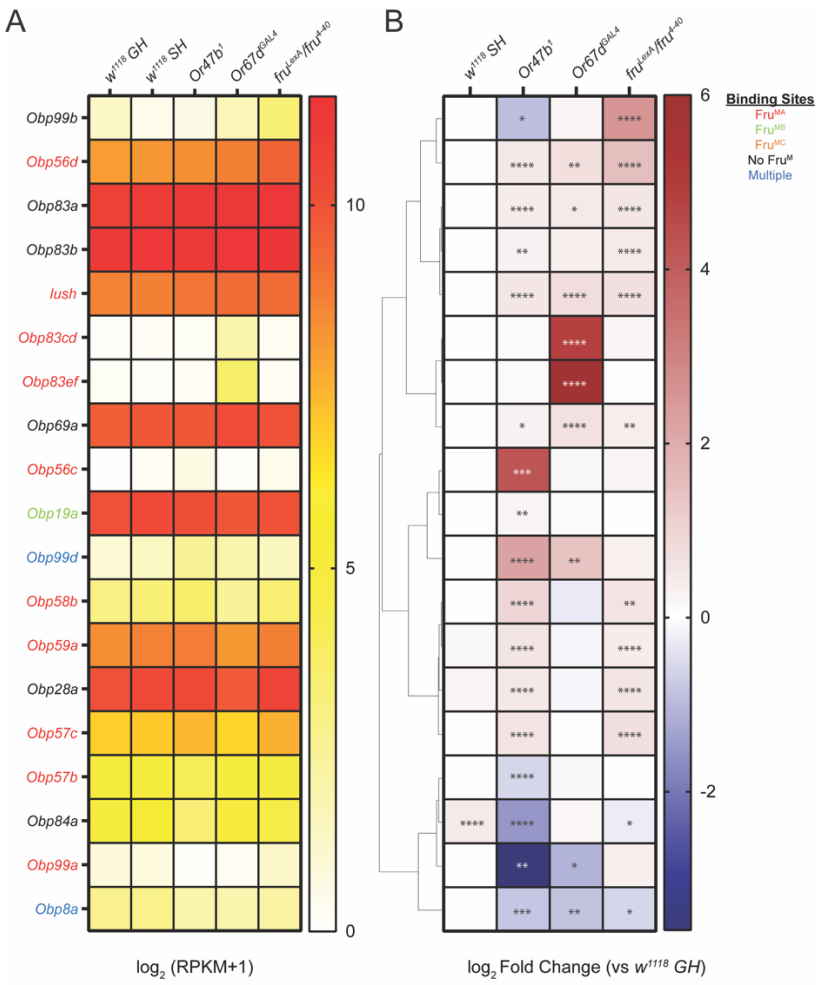


Figure 6-figure supplement 1. Validation of neurotransmitter receptor expression across ORN classes and in mutants. (A-B) ORN class-specific expression of serotonin and glutamate receptors based on single-cell RNA-seq datasets from the adult ORNs [66]**.** The size of each circle indicates the fraction of positive cells ($\log_2$(CPM+1)>0.5) and color intensity indicates the mean expression ($\log_2$(CPM+1)) of all positive cells. **(C-D)** Quantitative RT-PCR validation of *5-HT2A* **(C)** and *dmGlut* **(D)** expression from antennae of grouped and socially isolated wild types, *Or47b* mutants, *Or67d* mutants, and fru^M mutants normalized to *fl(2)d*.

468 ***Odorant binding proteins:***

469 Analysis of GO terms for molecular function for “odorant binding” highlighted genes encoding
470 odorant binding proteins (Obps) among the significantly altered compared to group housed wild
471 type male antennae (Figure 7). Previous studies using in situ hybridization and transcriptional
472 reporters have shown that Obps are generally produced in the non-neuronal support cells of the
473 antennal sensilla and are secreted into the local hemolymph [82]. However, our analysis of
474 previously published single-cell RNAseq data from ORNs [66] revealed that some Obps (i.e.
475 *Obp19a, Obp28a, Obp56a, Obp59a, Obp69a, Obp83a, Obp83b,* and *lush)* are abundantly
476 expressed in ORNs as well (Figure 7-figure supplement 1A). Odorants that enter the sensilla
477 through pores are thought to interact with Obps in the hemolymph, which aid with odor binding
478 to receptors on the cilia of ORNs [82]. Mutants in Obp genes are associated with alterations in
479 the spontaneous or evoked neuronal response dynamics of resident ORNs [82-87].
480 Analysis of Obp gene expression in the mutant male antennae showed many Obp transcripts
481 that normally are expressed in trichoid sensilla were increased in the antennae from *Or47b,*
482 *Or67d* and fru^M mutants (eg. *Obp83a, Obp83b, lush, Obp69a*) [82, 85-89] (Figure 7). qRT-PCR
483 from antenna generally corroborates RNA-seq results (Figure 7-figure supplement 1B,C).
484 Among the Obps that are differentially expressed in mutants, *Obp69a* is particularly interesting
485 as it was previously shown to modulate social responsiveness in Drosophila [90]. In this context,
486 cVA exposure in males as well as activation of Or67d neurons decreases *Obp69a* levels, which
487 in turn alters aggressive behaviors driven by Or67d neurons. In addition, *lush*, *Obp83a* and
488 *Obp83b* which are also expressed in trichoid sensilla were all shown to regulate odor evoked
489 response kinetics and spontaneous activity of trichoid ORNs [83, 85-87].
490 In addition to Obps expressed in the trichoid sensilla, many other Obps also show misregulation
491 particularly in *Or67d* and *Or47b* mutants. For example, in both mutants *Obp99d* is significantly
492 upregulated; in contrast, *Obp99a* and *Obp8a* show a downregulation (Figure 7). Even though it
493 is not known which sensilla these Obps are normally expressed in, given the responses it is

494 likely that they are produced by the non-neuronal cells in trichoid sensilla where Or47b and
495 Or67d ORNs are housed. There are also some Obps that show misregulation only in specific
496 mutants. For example, *Obp83cd, Obp83ef, Obp56c* are normally not expressed in the antennae,
497 yet *Obp83cd, Obp83ef* show a significant upregulation in *Or67d* mutants, whereas *Obp56c* is
498 upregulated in *Or47b* mutants (Figure 7). *Obp84a* is the only *Obp* to be upregulated in isolated
499 male antennae, and downregulated in *Or47b* mutant antennae (Figure 7). These results suggest
500 the presence of regulatory interactions between olfactory receptor signaling and neural activity
501 that likely drive activity-dependent homeostasis in Obp levels. Given the role of most Obps in
502 regulating neuronal physiology, it is possible that transcriptional changes in *Obp* genes
503 observed in social isolation as well as pheromone receptor mutants might occur as homeostatic
504 mechanism to compensate for altered neuronal activity and ORN function.
505



506

507 **Figure 7. Differentially expressed *Odorant binding protein (Obp)* genes in response to**

508 **social isolation, loss of pheromone receptors or *fru^M*. (A-B)** Many *Obp* genes show

509 significant changes. Hierarchically clustered heatmaps showing $\log_2$ fold change compared to

510 group-housed wild-type antennae across all experimental conditions **(B)** and average mRNA

511 levels (RPKM) of replicates within each condition ordered in the same way as $\log_2$ fold change

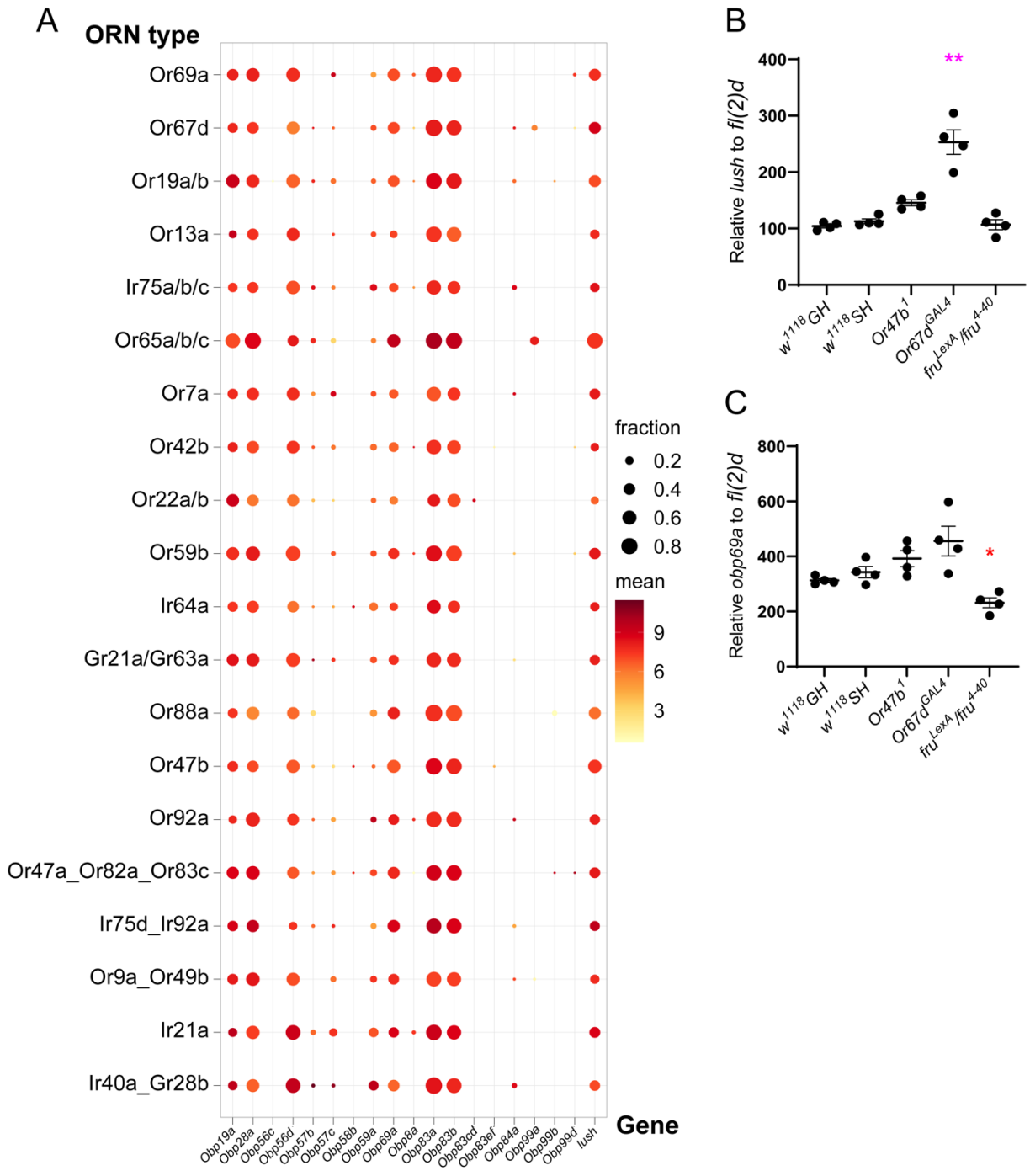
512 **(A)**. Genes with adjusted p-value above 0.01 were filtered out in each experimental condition.

513 Adjusted p-value was directly performed via DESeq2. *, p.adjust<0.05; **, p.adjust<0.01; ***,

514 p.adjust<0.001; ****, p.adjust<0.0001. Fru^M binding information is listed in the supplementary

515 file. Fru^M binding information is listed in Supplementary table 4.

516



517

518 **Figure 7-figure supplement 1. Validation of *Obp* expression across ORN classes and in**

519 **mutants. (A)** ORN class-specific expression of *Obps* based on single-cell RNA-seq datasets

520 from the adult ORNs [66]**.** The size of each circle indicates the fraction of positive cells

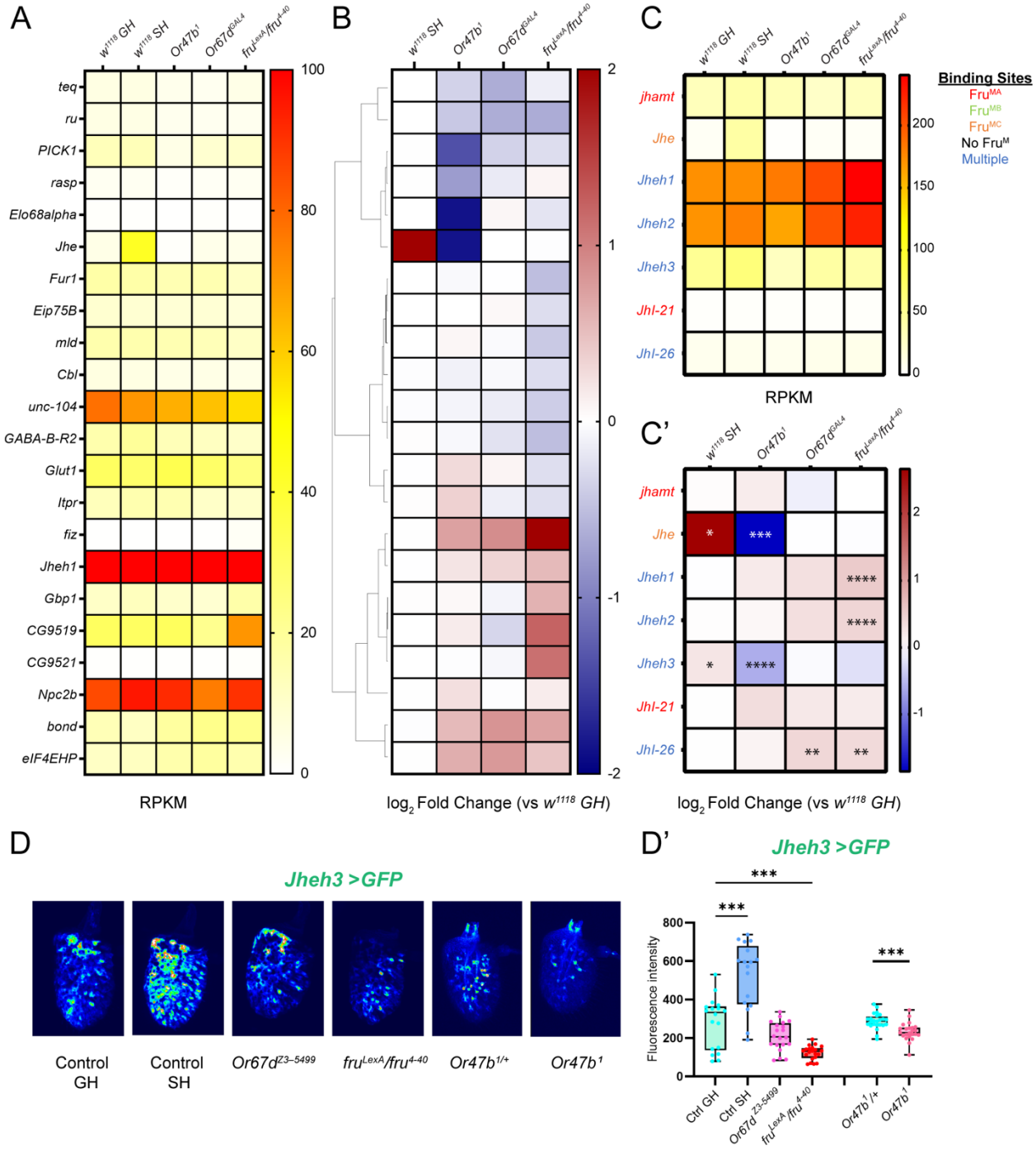
521 ($\log_2$(CPM+1)>0.5) and color intensity indicates the mean expression ($\log_2$(CPM+1)) of all

522 positive cells. **(B-C)** Quantitative RT-PCR validation of *lush* **(B)** and *Obp69a* **(C)** expression
523 from antennae of grouped and socially isolated wild types, *Or47b* mutants, *Or67d* mutants, and
524 fru^M mutants normalized to *fl(2)d*.
525

526 Pheromone receptor signaling regulates genes involved in hormone 527 metabolism

528 Hormone signaling is responsible for regulating behavioral and brain states in both vertebrates
529 and invertebrates. For example, in vertebrates many social behaviors such as aggression,
530 mating, and parenting, are under the control of hormones such as estrogen, testosterone,
531 oxytocin and vasopressin [91-94]. In social insects, such as ants, caste-specific behaviors are
532 determined by hormone states, where queen- and worker-like behaviors are associated with
533 ecdysone and juvenile hormone signaling, respectively [95, 96]. In Drosophila, juvenile
534 hormone signaling modulates behavioral and motivational states during courtship [20, 97, 98].
535 Recent studies have also identified age-related cues such as juvenile hormone (JH) signaling
536 together with social experience to control Or47b neuronal responses to pheromones and
537 courtship behaviors in a Fru^M-dependent manner [4, 20, 25]. JH signaling, concurrent with social
538 experience, modifies chromatin around *fru P1* promoter and ultimately fru^M levels in Or47b
539 ORNs [24]. These studies also demonstrated that JH receptor enrichment at *fru P1* promoter
540 increases in socially isolated flies as well as flies with disrupted Or47b signaling [24]. As
541 mentioned above, gene ontology analysis of differentially expressed genes in this study also
542 highlights genes involved in hormone metabolism (Figure 3). Thus, we specifically interrogated
543 the genes regulating hormone levels in pheromone receptor and fru^M mutants (Figure 8A-C').
544 Many of the enzymes involved in juvenile hormone biosynthesis and metabolism, such as
545 juvenile hormone epoxide hydrolases (*Jheh1*,*2*,*3*), juvenile hormone esterase (*Jhe*), and
546 juvenile hormone acid methyltransferase (*jhamt*), are expressed at varying levels in the

547 antennae (Figure 8C). These genes are also reported to have Fru^{MA} and Fru^{MC} binding in their
548 upstream regulatory elements (Dalton et al., 2013; Neville et al., 2014; Vernes, 2014). Two
549 mostly enriched genes, *Jheh1* and *Jheh2*, show mild upregulation in fru^{M} mutants but no
550 significant changes in the absence of social cues or pheromone receptor signaling (Figure
551 8C,C'). On the other hand, both *Jhe* and *Jheh3* appear to be upregulated in social isolation
552 while downregulated in *Or47b* mutants (Figure 8C'; Figure 8- figure supplement 1B).
553 Throughout the antenna, *Jheh3-GAL4* expression is observed in many ORNs (Figure 8D, D'). In
554 agreement with a transcriptional increase in socially isolated male antennae we found that
555 *Jheh3* reporter expression was significantly increased in isolated male antennae. On the other
556 hand, in both *Or47b* and fru^{M} mutant antennae *Jheh3* reporter expression was significantly
557 decreased in agreement with transcript levels (Figure 8D,D'). As observed in the RNA-seq,
558 there was no change in *Jheh3* reporter expression in *Or67d* mutants compared to grouped male
559 antennae (Figure 8D,D'). *Jhe* is of particular interest as Jhe activity is known to be necessary for
560 robust male-specific courtship behaviors and mating success in addition to affecting the
561 abundance of sex-specific pheromones such as 11-cis-vaccenyl acetate in males [36, 99].
562 Furthermore, seminal work on Jhe and Jheh3 has shown that these enzymes work together to
563 catabolize JH in *D. melanogaster* [100]. These results suggest that social experience and
564 pheromone receptor signaling regulates the expression of JH biosynthetic enzymes. Such
565 changes can modulate juvenile hormone activity by rapidly catabolizing JH in the periphery and
566 affecting downstream target genes, such as *fruitless*.
567



568

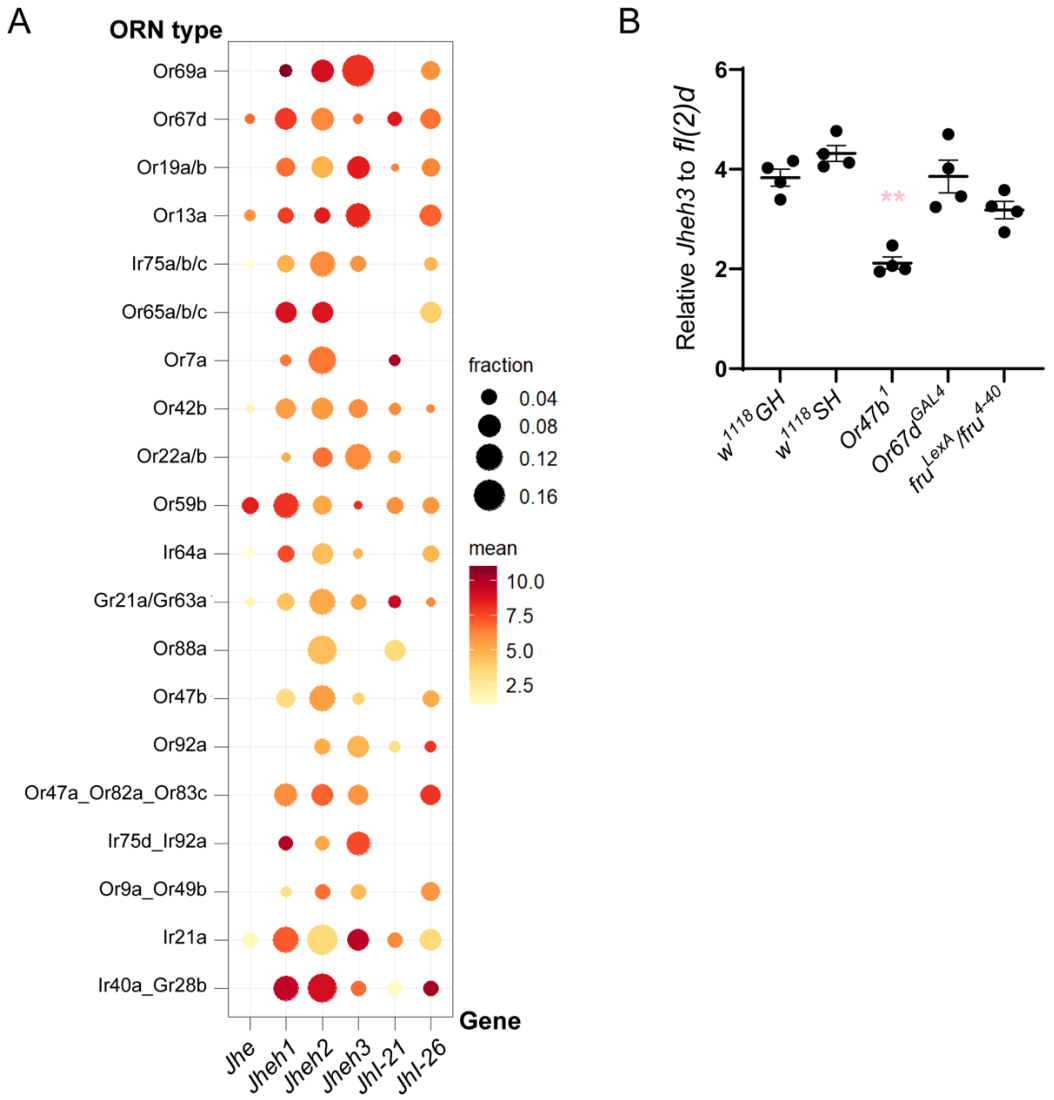
569 **Figure 8. Differentially expressed hormone metabolism genes in response to social**

570 **isolation and loss of pheromone receptor or *fru*^{M}. (A-B)** Examination of GO term: 0010817

571 (regulation of hormone levels) shows significant changes in various hormone metabolism gene

572 subclasses. Hierarchically clustered heatmaps showing $\log_2$ fold change compared to group-

573 housed wild-type antennae across all experimental conditions **(B)** and average mRNA levels
574 (RPKM) of replicates within each condition ordered in the same way as $\log_2$ fold change **(A)**.
575 Genes with adjusted p-value above 0.01 were filtered out in each experimental condition. **(C-C')**
576 RPKM **(C)** and $\log_2$ fold change **(C')** for juvenile hormone metabolism-related genes. Adjusted
577 p-value was directly performed via DESeq2. *, p.adjust<0.05; **, p.adjust<0.01; ***,
578 p.adjust<0.001; ****, p.adjust<0.0001. Fru^M binding information is listed in Supplementary table
579 4. **(D)** Confocal images of antenna from WT GH, WT SH, *Or47b*^{1}, *Or67d*$^{Z3\text{-}5499}$, and *fru*$^{LexA/4\text{-}40}$
580 mutant males expressing *Jheh3-GAL4 UAS-mCD8GFP.* **(D')** Quantification of fluorescence in
581 images presented in D. **(D-D')** n=17-24.
582



583

584 **Figure 8--figure supplement 1. Validation of hormone-related gene expression across**

585 **ORN classes and in mutants. (A)** ORN class-specific expression of juvenile hormone

586 regulators based on single-cell RNA-seq datasets from the adult ORNs [66]. The size of each

587 circle indicates the fraction of positive cells ($\log_2$(CPM+1)>0.5) and color intensity indicates the

588 mean expression ($\log_2$(CPM+1)) of all positive cells. **(B)** Quantitative RT-PCR validation of

589 *Jheh3* expression. Relative expression of *Jheh3* from antennae of grouped and socially isolated

590 wild types, *Or47b* mutants, *Or67d* mutants, and fru^M mutants normalized to *fl(2)d*.

591

592 # Discussion

593 Sensory experience influences many behaviors by modifying neuronal and circuit function [1-4],
594 yet, molecular mechanisms remain largely unknown. Here, we took advantage of the well-
595 characterized system of sex-specific behaviors, governed by the Fru^M, which acts as a gene
596 regulatory switch for male-specific circuit development, function, and behavior in *Drosophila*
597 *melanogaster* [10, 11, 15, 101]. Our results show that social experience and pheromone
598 signaling alters Fru^M splicing patterns and neuromodulatory gene expression programs, which
599 ultimately modulate circuit function and behavioral responses (Figure 9).
600 Previous studies in Drosophila demonstrated that social experience can modulate Fru^M-
601 dependent sex-specific behaviors such as courtship and aggression [1, 3, 4]. For example,
602 social isolation decreases the sensitivity of Or47b neurons to their pheromone ligands in a Fru^M-
603 dependent manner, which leads to a decrease in male competitive courtship advantage
604 [4]. Other studies have also shown that monosexual group housing can decrease aspects of
605 courtship behaviors such as courtship song and circling [102]. In addition to courtship,
606 aggression behaviors which are under the control of Or67d and Or65a neurons and Fru^M
607 function, also change with social experience [40, 102]. For example, social isolation significantly
608 increases male-male aggression [33, 102]. These reports highlight the importance of social
609 experience and pheromone signaling in the execution of sex-specific behaviors.
610 What are the molecular mechanisms by which Fru^M function is altered by social experience? We
611 previously reported that social experience and pheromone receptor signaling alter chromatin
612 states around *fru* P1 promoter [24] to modify *fru* transcription [4, 23, 24]. Nonetheless, the
613 consequential details of chromatin-based changes on *fru* transcription remained unknown. One
614 of our most intriguing findings from this transcriptome analysis is that these chromatin effects
615 are associated with changes in the relative exon usage and splicing patterns of *fru* gene in
616 response to impaired pheromone detection (Figure 4). Transcriptional regulation of *fru* is

complex yielding 15 annotated alternatively spliced isoforms from 7 promoters giving rise to different 3' sequences which encode variable Zinc-finger DNA-binding domains of Fru protein [13, 27, 103, 104]. Different Fru proteins regulate unique yet overlapping set of target genes which have binding sites for single or multiple Fru^M isoforms [27, 29, 62]. Many of these target genes regulate neural development and function. Therefore, changes in *fru* splicing patterns can affect the expression of thousands of genes simultaneously, strongly modulating neuronal responses and circuit outputs in a short period of time. Remarkably, Fru^M is expressed in ~2000 interconnected neurons highlighting a circuit for courtship behaviors from sensation to action [11, 105]. This expression pattern allows neural activity-dependent influences on *fru* chromatin and transcription to propagate throughout the whole circuit. In summary, these features make circuit switch gene fru^M an efficient molecular hub onto which many internal and external states act to modulate circuit activity and behavioral outputs by tweaking the levels of transcripts and splice isoforms, leading to a cascade of changes in transcriptional programs.

Each pheromone sensing neuron relays different information about the social environment, which is integrated and processed to output a specific behavior. Likely due to differences in neuronal identity and function, different pheromone receptors have different effects on *fru* chromatin and splice isoforms [24] (Figure 4). Such sensory stimuli-dependent changes in Fru proteins can alter the expression of downstream neuromodulatory genes to have rapid, temporary, or lasting effects on neuronal activity and behavioral outputs. These changes are essential for organisms to form short/long-term adaptation to the environment. However, how these different cell types generate these differences in behavioral repertoire via changes in gene expression in the periphery have been largely unknown.

Many of the genes that show differential expression in response to social isolation and disruption of pheromone receptor or Fru^M function encode neuromodulators that affect membrane potential, such as ion channels, membrane ion transporters, proteins involved in neurotransmission, and odorant binding proteins (Figure 3; Figure 5; Figure 6). Among all

643 conditions, social isolation possesses the fewest differentially expressed genes compared to
644 group-housed controls with a small overlap with pheromone receptor and *fru*^{M} mutants. This
645 might be due to differences in gene expression changes in response to disruption of evoked
646 activity of pheromone sensing olfactory neurons with socially isolation versus disruption of both
647 spontaneous and evoked activity in pheromone receptor mutants. Loss of Fru^{M} alters the
648 expression of many neuromodulatory genes with known Fru^{M} binding sites in a bimodal way,
649 suggesting Fru^{M} can act as both an activator and repressor of gene expression. Some of these
650 differentially expressed genes are also altered in pheromone receptor mutants, generally in the
651 same direction (Figure 2D,E). There are also unique overlaps between *Or47b* and *fru*^{M} mutants,
652 between *Or67d* and *fru*^{M} mutants, and between *Or47b* and *Or67d* mutants (Figure 2B,E). Many
653 of these differentially expressed genes are known to harbor binding sites for different Fru^{M}
654 isoforms. These suggest that some of the differentially expressed genes in *Or47b* and *Or67d*
655 mutant are due to Fru^{M}-dependent changes, whereas others might be Fru^{M}-independent,
656 caused by OR signaling and/or ORN activity.
657 One functionally relevant gene among the genes that show differential regulation in pheromone
658 receptor and *fru*^{M} mutants is the Fru^{M} target gene *ppk25*, which previously was shown to
659 modulate ORN responses in Or47b and Or67d neurons [25, 26]. *ppk25* belongs to a family of
660 sodium channels that serve a variety of functions, from neuromodulation to detection of sensory
661 cues. PPK protein complexes, generally are composed of multiple subunits encoded by different
662 *ppk* genes. Many *ppk* genes contain binding sites for Fru^{M} isoforms in their promoter regions
663 [27, 29, 62]. In addition, a recent study implicated isoform-specific Fru^{M}-dependent regulation of
664 *ppk25* and *ppk23* in the modulation of Or47b and Or67d responses [25, 26]. According to the
665 genetic analysis in this study, Fru^{MB} and Fru^{MC} positively regulate the expression of *ppk25* and
666 *ppk23*, respectively. There are apparent discrepancies with this interpretation and transcriptome
667 data from our study, as well as others [66, 106]. While our transcriptome analysis agrees with a
668 regulatory role for Fru^{M} in *ppk25* gene regulation, the regulatory mode is repressive; that is,

669 *ppk25* expression is upregulated in *Or47b*, *Or67d,* and *fru* mutants. This type of repressive role
670 for Fru^M in transcription also is in consensus with previous studies demonstrating Fru^M
671 interactions with transcriptionally repressive histone-modifying enzymes such as HDAC1 [48,
672 107]. In addition, we are not able to detect any transcripts for *ppk23* in the antennae, and the
673 expression of *ppk23* does not change in *Or47b*, *Or67d,* and fru^M mutants. Instead, we noticed
674 other *ppk* genes such as *ppk6*,*7*,*13*,*14*,*15*,*19* are altered in different mutant conditions. Fru^M
675 seems to have a bidirectional role in regulating *ppk* gene expression, where it activates the
676 expression of a subset of *ppk* genes (*ppk7*,*13*,*14*,*15*) while repressing the expression of others
677 (*ppk6* and *ppk25*). One way to reconcile these differences is that multiprotein PPK complexes
678 composed of combinations of different PPK subunits, and the stoichiometric levels of each *ppk*
679 transcript in a given neuron can determine channel function. For example, misexpression of
680 *ppk23*, which normally is not expressed in the antennal ORNs, can interfere with PPK channel
681 function by disrupting the existing functional complexes in a given neuron, or forming new PPK
682 complexes, thus affecting physiological properties. Another possibility is that the transcriptional
683 changes in $fru^{Lex}/fru^{/4-40}$ mutant are an output for eliminating all fru^M transcripts, thus masking
684 individual effects of each fru^M isoform, such as fru^{MA}, fru^{MB}, *or* fru^{MC}. And finally, it is also
685 possible that the slight upregulation of *ppk25* in *Or47b* and fru^M mutants as well as large
686 changes in *Or67d* mutants may be due to global fru^M changes in the whole antennae, or through
687 retrograde neuromodulatory signaling from the antennal lobe.
688 Antennal sensilla contain cell types other than ORNs, such as glia-like cells and support cells of
689 sensillum, as well as epithelial cells. Since our transcription data is from the whole antennae,
690 one possibility we cannot exclude is that differences in antennal gene expression in different
691 genetic and social conditions are readouts from non-neuronal cells or other ORNs. Even though
692 we anticipate the immediate effects of *Or67d* and *Or47b* mutants to happen in the ORNs
693 expressing these two receptors, signals from ORNs can lead to secondary changes in gene
694 expression in non-neuronal cells within the sensillum. This also brings to light a general issue

695 with bulk tissue where large cell-type-specific changes may be masked by cell-nonautonomous
696 changes in gene expression in others cell types, as well as retrograde feedback signaling within
697 olfactory circuits. Regardless, our data shows many of the differentially expressed genes
698 encode regulators of neuronal function and neuromodulation. This increases the likelihood that
699 the transcriptional changes in response to social and pheromonal cues are happening mostly in
700 the neurons that respond to social cues, such as Or47b and Or67d ORNs. Future single-cell
701 chromatin and transcription profiles from Fru^M-positive neurons in the antenna and brain will
702 provide deeper insights to neuron-specific changes in gene regulation from the peripheral to the
703 central nervous system that modulate circuit function in response to social cues.
704 Part of the transcriptional effects can also be exerted downstream of changes in *dsx* levels seen
705 in pheromone receptor and *fru* mutants. Given the upregulation of *dsx* levels in pheromone
706 receptor and *fru* mutants suggests the possibility that some of the social experience- and neural
707 activity-dependent transcriptional changes might also arise from increased Dsx. Dsx expression
708 is restricted to non-neuronal cells in the antenna [58]. Similarly, neuromodulatory genes such as
709 *Obps* and some neurotransmitter receptors, which function to alter both spontaneous and
710 evoked activity of ORNs, are also expressed in non-neuronal cells in addition to the ORNs in the
711 antennae [82-84, 88]. The social experience and pheromone signaling-dependent misregulation
712 of these genes, points to adaptive homeostatic mechanism within local sensilla that can
713 contribute to modulation of neuronal activity.
714 Lastly, in addition to the transcriptional changes occurring in neuromodulatory programs, genes
715 regulating juvenile hormone metabolism are also modified with social context and pheromone
716 receptor and *fruitless* mutants. Social experience works together with juvenile hormone
717 signaling to modulate responses of pheromone sensing neurons in a Fru^M dependent manner
718 [4]. These contribute to modification of competitive copulation advantage of males in different
719 population densities and different ages as well as regulating overall courtship. At the molecular
720 level social/pheromonal cues work together with juvenile hormone receptors to modulate

721 chromatin around *fruitless* P1 promoter and its transcription [24]. Juvenile hormone acts as a
722 repressor of *fru* expression and social experience converts it to an activator. In the same study,
723 we showed that social isolation and disruption of Or47b signaling increases the accumulation of
724 juvenile hormone receptor at the *fru* P1 promoter and juvenile hormone response elements.
725 This might be due to changing levels of juvenile hormone since our results show that expression
726 of genes involved in juvenile hormone metabolism are altered in social isolation, and mutants in
727 pheromone receptors and *fru*. The findings in our study together with results from previous
728 studies suggest the presence of interconnected gene regulatory networks among
729 social/pheromone signaling, hormone signaling and Fru^M function in neural and behavioral
730 modulation (Figure 9).
731

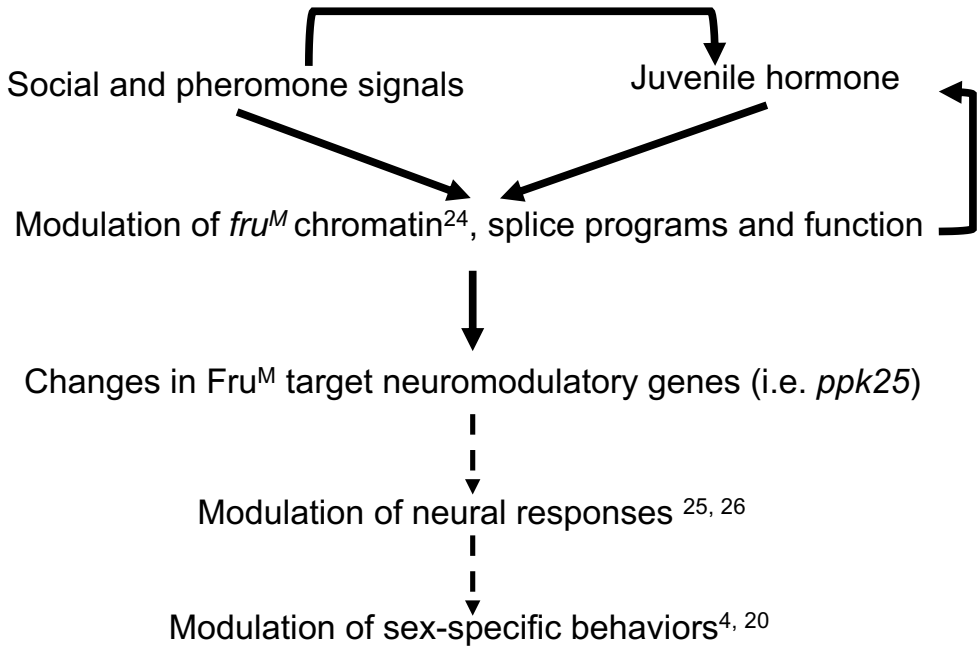


Figure 9. Fruitless-dependent transcriptional cascade that reprograms neural responses and behaviors with social experience, pheromone receptor function and hormone signaling. Social context and pheromone detection modifies chromatin [24] and transcriptional/splice programs for *fruitless* gene altering its function. This reprograms expression of Fruitless target neuromodulatory genes (i.e. *ppk25*) altering neural physiology and pheromone responses [4, 25, 26]. Ultimately, these result in changes in neuronal activity and behavioral modulation [4]. It was also shown that juvenile hormone signaling works together with social experience to modulate both ORN physiology and courtship behaviors [20, 25]. At the molecular level social/pheromonal cues work together with juvenile hormone receptors to modulate transcription *fruitless* [24]. Social context, pheromone receptor, and Fru^{M} function also alter the expression of genes involved in juvenile hormone metabolism.

745

746 # Conclusions

747 Social isolation is known to affect a wide range of brain functions and behaviors, such as
748 aggression, attention, depression, and anxiety. Overall, this study highlights the shared
749 transcriptional changes in master behavioral regulators and their target neuromodulatory genes
750 providing a molecular mechanism that alter neural responses with social experience and
751 pheromone sensing.

752

Material and Methods

Fly genetics and genotypes

Flies were raised on standard fly food (containing yeast, cornmeal, agar, and molasses) at 25°C in a 12-hour light/12-hour dark cycle in cylindrical plastic vials (diameter, 24 mm and height, 94 mm). For social isolation (single housing, SH) condition, 80-100 hour-old pupae were separated by sex and males were placed into individual vials, allowed to eclose alone, and aged to 7 days to deprive flies of pheromone interaction on ORNs. For group housing (GH) condition, 25-30 newly eclosed males were collected and placed into food vials. These were aged to 7 days and 180 antennae were dissected per sample, for a total of 3 samples for w^{1118} *GH*, 3 samples for w^{1118} *SH*, 3 samples for $Or47b^{1}$ mutants (*$Or47b^{1}$/$Or47b^{1}$; Or47b-GAL4, UAS-mCD8GFP/ Or47b-GAL4, UAS-mCD8GFP*), 3 samples for $Or67d^{GAL4}$ mutants (*UAS-mCD8GFP/ UAS-mCD8GFP; $Or67d^{GAL4}$/$Or67d^{GAL4}$*), and 2 samples for fru^{LexA}/ fru^{4-40} mutants (w^{+}; +/+; fru^{LexA}/ fru^{4-40}).

766 **Table 1. Genotypes used in in-vivo validation of expression.**

Figure	Type	Genotype	Source
Figure 6G,G'	Control	*+/+; 5-HT2A[2048-GAL4]/40XUAS-mCD8GFP*	*5-HT2A[2048-GAL4]*: BDSC 66185
	Or47b^{1}	*Or47b^{1}/ Or47b^{1}; 5-HT2A[2048-GAL4]/40XUAS-mCD8GFP*	
Figure 6H,H'	Control	*dmGlut[0546-GAL4]/UAS-mCD8GFP; +/+*	*dmGlut[0546-GAL4]*: BDSC 63397. *Or67d$^{Z3-5499}$*: Dean Smith Lab, UT Southwestern
	Or67d$^{Z3-5499}$	*dmGlut[0546-GAL4]/UAS-mCD8GFP; Or67d$^{Z3-5499}$/Or67d$^{Z3-5499}$*	
	fru^{LexA}/fru$^{4-40}$	*dmGlut[0546-GAL4]/UAS-mCD8GFP; fru^{LexA}/fru$^{4-40}$*	
Figure 8D,D'	Control	*Jheh3[0892-GAL4]/UAS-mCD8GFP; +/+*	*Jheh3[0892-GAL4]*: BDSC 63877. *Or67d$^{Z3-5499}$*: Dean Smith Lab, UT Southwestern
	Or67d$^{Z3-5499}$	*Jheh3[0892-GAL4]/UAS-mCD8GFP; Or67d$^{Z3-5499}$/Or67d$^{Z3-5499}$*	
	fru^{LexA}/fru$^{4-40}$	*Jheh3[0892-GAL4]/UAS-mCD8GFP; fru^{LexA}/fru$^{4-40}$*	
	Or47b^{1}/+	*Or47b^{1} Jheh3[0892-GAL4]/+; 40XUAS-mCD8GFP/+*	
	Or47b^{1}	*Or47b^{1} Jheh3[0892-GAL4]/Or47b^{1}; 40XUAS-mCD8GFP/+*	

767

768

769 ## RNA-seq

770 RNA-seq was performed as described before (31). Male flies are aged for 7 days and dissected
771 for the third antennal segment (~180 antennae per genotype). RNA was extracted from
772 dissected tissues samples using Qiagen RNA-easy extraction kit, quantified using a Qubit RNA
773 assay kit and checked for quality using a High Sensitivity RNA ScreenTape on a TapesStation
774 (Agilent). RNA integrity scores are typically 7.0 and greater. 1ug of RNA was used to construct
775 libraries for sequencing using a KAPA mRNA library prep kit with polyA RNA selection.
776 Barcoded libraries are sequenced on a Novaseq 6000 SP 50 bp following manufacturer's
777 instructions (Illumina). After demultiplexing sequence quality was assessed using FASTQC
778 (Version 0.11.9). While there are issues with under-clustering of the samples and unbalanced
779 pools, the data quality was typical for RNA extracted from fresh frozen material. The unbalanced
780 pools resulted in differences in sequencing depth of each sample.
781

782 ## Analysis of RNA-seq data

783 Once sequenced, the reads are preprocessed with FASTP [108] to remove adaptors and
784 trim/filter for quality. These are mapped to the dm6 reference genome using MapSplice2 [109],
785 with individual mapping rates exceeding 98% in all cases. This raw alignment was deduplicated
786 and filtered for mapping quality and correct pairing; additional alignments are generated to
787 confirm results are robust to mapping ambiguity. Mapped reads are assigned to genes in the
788 annotation using the feature Counts command from the SubRead package [110]. Differential
789 expression was modeled using DESeq2 [111] using the "apeglm" shrinkage estimator, and data
790 was processed and visualized in R using the tidyverse framework [112], supplemented with the
791 biomaRt [113], ComplexHeatmap [114] and UpSet [115] packages. The bioinformatics pipeline
792 was implemented in Snakemake [116]. Code for the analysis is deposited on GitHub
793 (https://github.com/csoeder/VolkanLab_BehaviorGenetics/tree/master/scripts).

794 DEXSeq was used to test for differential exon use under models corresponding to those used in
795 differential gene expression [46]. From the genome-wide test, the *fruitless* locus was examined
796 in particular.
797

798 Statistical analysis

799 Adjusted p-value were directly calculated from DESeq2 or DEXSeq (Supplementary table 1).
800 Other statistical analysis is described in the legend of corresponding figures.
801 Specifically, to compare the exon usage in Figure 4, we also calculated p-value from post-hoc t-
802 tests from raw read counts of independent comparisons of group-housed male antennae to
803 each experimental condition at an individual exon segment (1-22, see the table 2 below). Even
804 though many exons level differences were significant using this method, adjusted p-value from
805 DEXSeq gave rise to fewer significantly altered exon levels.
806

807 **Table 2. t-test p-value of *fru* exons from DEXseq.**

t-test p-value	*w^{1118} GH* vs			
Exon	***w^{1118} SH***	***Or47b^{1}***	***Or67d^{GAL4}***	***fru^{LexA}/fru$^{4-40}$***
P1 (1)	0.5696	0.0316	0.0619	0.0764
Male (2)	0.6843	0.0292	0.0125	0.0013
Female (3)	0.0697	0.3486	0.6932	0.5993
P2 (4)	Expression too low to calculate			
P6 (5)	Expression too low to calculate			
P3 (6)	Expression too low to calculate			
Exon 7 (7)	Expression too low to calculate			
Exon 8 (8)	Expression too low to calculate			
PD (9)	Expression too low to calculate			
P4 (10)	0.8788	0.0387	0.3002	0.1493
P5 (11)	Expression too low to calculate			
C1 (12)	0.5489	0.0117	0.1050	0.0498
C2 (13)	0.6455	0.0826	0.0007	0.0079
C3 (14)	0.3622	0.1247	0.0399	0.0650
C4 (15)	0.8295	0.3811	0.0704	0.1205
D (16)	0.7090	0.2796	0.2787	0.7042
C5 (17)	0.7575	0.7338	0.2378	0.3174
3'UTR (18)	0.7235	0.0086	0.0754	0.1328
FruA (19)	0.5241	0.0498	0.0171	0.0878
FruB (20)	0.8142	0.4809	0.7873	0.2550
FruMC male (21)	0.8519	0.1819	0.1969	0.1943
FruFC female (22)	0.4168	0.1123	0.8214	0.0223

808

809 ## Quantitative reverse transcription PCR (qRT-PCR)

810 The qRT-PCR protocol was modified based on the previous protocol of the Volkan lab [32]. For
811 each genotype (same as RNA-seq), four biological replicates were prepared separately, with
812 each replicate containing 100 antennae from 50 males (7-day old). Antennae were dissected on
813 Flypad and transferred into TRIzol (Invitrogen, 15596026) immediately. Total antennae RNA
814 was extracted using the RNeasy Mini Kit (QIAGEN, 74104) and treated with Dnase I (TURBO
815 DNA-free Kit, Invitrogen, Thermo Fisher Scientific AM1907) to remove genome DNA. cDNA was
816 generated from the reverse transcription of 80-150ng total RNA using the SuperScript IV First-
817 Strand Synthesis Kit (Invitrogen, 18091050) and poly d(T) as transcription primers. qPCR was
818 performed using the FastStart Essential DNA Green Master kit (Roche, 06924204001) on
819 LightCycler® 96 instrument (Roche, 05815916001). Primers used are listed in Table 3 below.
820 The expression level was calculated by ΔCt method using the *fl(2)d* as the standard gene. The
821 calculation was performed in GraphPad Prism software. One-way ANOVA was used for
822 significance test, followed by multiple comparisons (compare other groups to group-housed wild
823 types w^{1118} *GH*). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

824

825 **Table 3. Primers sets used in qRT-PCR assays.**

Primer Names **(F: Forward; R: Reverse)**	**Sequences**
fl(2)d set15 F (exon spanning)	AGAAATCGCAGTCGGAGTT
fl(2)d set15 R	CCTTCTCAAGCGTTTGTATGC
fruM F (exon spanning)	CCCGCATCCCCTAGGTACAA
fruM R	GACTGTTTCGCCCTCGCAGG
dsxM F	GAGCTGATGCCACTCATGTAT
dsxM R	CTGGGCTACAGTGCGATTTA
wkd set34 F	AATGTGCTAAAGGCCTACTC
wkd set34 R (exon spanning)	TGCAGGTATACATCGCACA
ppk25 set11 F (exon spanning)	CTGCAGTATTACAGTCCCTACC
ppk25 set11 R	TCCGGATACTGTGCAGATTG
5-HT2A set15 F (exon spanning)	CCGTTCTTGGTCTGGTCAAT
5-HT2A set15 R	CGTCAATGCGTATGTGGTAAC
dmGlut set45 F	TCCTGAATGCCTACACGATG
dmGlut set45 R (exon spanning)	CACCAACACTGGTTCCCT
Jheh3 set10 F (exon spanning)	GACCGAAATTCAGGGCTTG
Jheh3 set10 R	GGTTAGCATGGGTATAAAGTCG
Lush set4 F (exon spanning)	CTTGTCGGGATACGCATAAA
Lush set4 R	TAAGGCCACATGAACTGC
Obp69a set6 F (exon spanning)	CAGGAGCTTCTGTAGATGTG
Obp69a set6 R	CTCCAACAGTGCTTCCAA

826

827

828 ## In-vivo validation of gene expression

829 Fly heads were dissected in cold PBT (phosphate buffered saline with Triton X-100) buffer and
830 were fixed in 4% PFA on nutator at room temperature for 1 hour. Fly heads were washed three
831 times with fresh PBT, and every wash was 10 min at room temperature. Antennae were
832 dissected from heads and were fixed in 4% PFA on nutator at room temperature for 30 minutes.
833 Antennae were washed three times with fresh PBT, and every wash was 10 min at room
834 temperature. Antennae were mounted using Fluoromount-G Slide Mounting Medium
835 (SouthernBiotech). Images were taken by Olympus FluoView FV1000 confocal microscope.
836 Controls and experimental groups were imaged by same parameters. Native fluorescence was
837 measured by ImageJ. The fluorescence intensity was defined as the fluorescence of region of
838 interest subtracted by that of the background. Statistical tests were performed in GraphPad
839 Prism software. One-way ANOVA was used for significance test, followed by multiple
840 comparisons (compare other groups to group-housed control). * $p<0.05$, ** $p<0.01$, *** $p<0.001$,
841 **** $p<0.0001$.
842
843

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Contributions

Conceptualization/design of the work: BD and PCV. Investigation: CD, QD, and BD with help from AM and DG. Analysis/interpretation of data: QD, BD, CD, CS, CDJ, and PCV. Drafted the work: BD, QD, CD and PCV. The authors read and approved the final manuscript.

Ethics declarations

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All relevant data are within the paper and its supporting information files. Raw sequencing data will be uploaded to the GEO under embargo pending the submission and acceptance of the data submitted here for publication.

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